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THE METABOLISM OF ETHYL ALCOHOL

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HISTORICAL

The great *Liebig* assumed that alcohol was oxidized in the human and animal organism through aldehyde, lactic acid, oxalic acid and formic acid to carbonic acid, but so far as is known he had no experimental evidence for his assumption. About 1860 the first animal experiments showed that ingested alcohol could be recovered qualitatively from the urine and the expired air, which made these early investigators conclude that alcohol was excreted from the body and not metabolized at all. This was the state of affairs when the first period of modern study of alcohol metabolism began about 1870.

Dupré (1872) (44) and *Anstie* (1874) (5) showed definitely that the alcohol recovered from the excretions over a long period was only an insignificant part of the alcohol administered, and that the rest disappeared. The German and Italian investigators *Binz* (14), *Schmidt* (134) and *Albertoni* (3) independently found the same. As it was impossible to demonstrate the presence of any metabolic products of alcohol in the body or in the excretions, it was concluded that 95–98 per cent of the ingested alcohol was totally oxidized in the organism to carbon dioxide and water.

The chemical energy liberated by the total oxidation of 1 gram alcohol is 7.1 calories, and in the years following 1880 much work was devoted to the question whether ingested alcohol is metabolized independently of the general metabolism. Experiments by *Henrijean* (1883) (71) and *Bodländer* (1883) (17) showed clearly that the general metabolism was not increased by the ingestion of alcohol. This was later confirmed by extensive and convincing experiments by *Bjerre* (1899) (15), *Atwater and Benedict* (1902) (7) and others. Moreover *Strassmann* (1891) (139) demonstrated that the bodies of animals given alcohol over a long period show a higher content of fat tissue than control animals kept on quantitatively and qualitatively the same diet but without alcohol. There was some discussion about the protein-saving effect of alcohol, but when it was discovered that the toxic action of alcohol at first causes an increased excretion of nitrogen in individuals not accustomed to alcohol, it was easy to show that alcohol spares protein to the same extent as isodynamic amounts of carbohydrate or fat. Thus the first period of the study of alcohol metabolism ended at the beginning of this century with the general acceptance of the very important fact that *alcohol can be utilized as a foodstuff by the organism.*

A little after 1900 the second period started with the study of the metabolic rate of alcohol. In 1903 *Gréhant* (61) analyzed whole animals at various times after the ingestion of alcohol. *Rosemann* (1909) (132) tried to calculate the metabolic rate in man from the depression of the respiratory quotient during alcohol oxidation, and in 1919 appeared *Mellanby's* (105) very commonly cited experiments in dogs. These and other investigators with their rather primitive analytical methods reached figures not deviating much from those found later. The literature up to 1904 is collected by *Abderhalden* (1) and the results of the studies up to 1920 are extensively reviewed by *Kochmann* (87) and *Rosemann* (133). *Widmark's* introduction of his micromethod for alcohol determination in 1922 (145) was an important step forward. During the following decade, stimulated by the increasing forensic importance of test on drunken drivers, by means of this and similar micromethods *Widmark* himself and other investigators cleared up the general facts about alcohol absorption, distribution and elimination in the human and animal body.

Our knowledge in 1935 can be summarized as follows: Alcohol is readily absorbed in the intestinal tract, but certain foodstuffs, especially protein and fat, are able to delay the absorption considerably. The distribution of alcohol in the body follows the equation given by *Widmark* (147):

$$A = p \times c \times r$$

A being the total amount of alcohol in the organism in grams, p the body weight in kg, and c the alcohol concentration in mg per gm blood. The factor r is the proportion of the body in which the alcohol is distributed; on the average it is $67\% \pm 20\%$, but it is relatively constant in the same individual from time to time. From the slope of the alcoholaemic curve and the formula it is possible to calculate that an average individual is able to oxidize about 1 gm alcohol per hour per 10 kg bodyweight. The results from these earlier periods are beyond dispute and well described in every textbook and will be omitted from this review.

Three general lines have been followed in the recent study of alcohol metabolism: (i) The factors that may influence the rate of alcohol metabolism and its connections with the metabolism of other compounds in the body; (ii) the sites of alcohol metabolism in the organism; (iii) the enzymes involved in alcohol metabolism. These lines will also be followed in the present review, not because they represent a chronological development or because they represent the most logical arrangement, but because the gap between the investigators who follow the different lines is often so wide that it is impossible to present the experimental results otherwise without imposing on authors opinions and interpretations that they never intended. The following earlier reviews can be recommended which give different points of view on the question,—***Widmark's*** **(1932,** in German) (146), ***Kochmann's*** (1936, in German) (88), ***Le Breton's*** (1936, in French) (94), *M. Schmidt's* (1937, in Danish, 135), ***Jellinek and Jolliffe's*** **(1940,** in English, (78), *Carpenter's* (1940, in English) (21), ***Derobert and Duchène's*** (1942, in French) (35), and *Newman's* (1941 and 1947, both in English) (111, 112).

ALCOHOL METABOLISM IN INTACT ORGANISMS

Most investigators working on this problem have measured the rate of disappearance of alcohol from the blood after the administration of alcohol, either intravenously or more commonly by mouth to the experimental subjects. On many points there is much disagreement. One reason is that the technique of many of the investigators has not been beyond criticism. Far too many have disregarded the rather high variation in the absorption of alcohol from the intestinal tract and have not realized that the time required to obtain complete equilibrium in the distribution of alcohol in the tissues is rather long even when the alcohol is given intravenously. The reviewer regrets that only too few of the numerous published experiments fulfill the requirements of a technique which cannot be criticized; the best to say about the rest is that they sometimes are able to support the results obtained by better methods, but not always. In any case they will never be able to contradict them.

The Slope and Shape of the Alcoholaemic Curve in the Postabsorptive Period: Since the work of *Mellanby* (105) and *Widmark* (146) it has generally been accepted that the decrease of the alcohol content in the organism, as measured by the concentration in blood, follows a straight line from the point where the absorption is ended and a diffusion equilibrium between the tissue fluids and the tissues has been established until all measurable remnants of alcohol have disappeared from the blood. The most common expression of this slope is found in *Widmark's* factor, β, which indicates how many milligrams of alcohol disappear from each gram (or ml) blood per minute or per hour. The β factor is independent of the concentration of alcohol in the blood. Since then numerous observations in many species have confirmed these fundamental observations, sometimes even called "laws."

In spite of this, the conclusion that the metabolic rate of alcohol is independent of the concentration of alcohol in the organism has been repeatedly challenged, and convincing experimental evidence has been brought against it. Not a few investigators have failed to find a rectilinear curve of alcohol disappearance from the body and found a hyperbolic curve instead, the slope of which decreases with decreasing concentrations of alcohol. Even some who have found a rectilinear course of the curve claim a definite dependence of alcohol metabolism on alcohol concentration. *Newman et al.'s* (122) experiments have led to the astonishing conclusion that the metabolic rate of alcohol depends on the initial content of alcohol in the organism, but remains constant until all alcohol has disappeared. The experiments do show that a dog starting with a higher alcohol content in the organism has a steeper decline in the alcoholaemic curve than another dog starting with a lower alcohol content, even when the decline is measured from a point where the two dogs have exactly the same concentration of alcohol in their blood. Moreover, if a dog is pretreated with a certain amount of alcohol, a renewed smaller dose given shortly after the first dose of alcohol has been metabolized will disappear at a constant rate equal to the rate of disappearance of the first dose (122). *Newman* says that the high dose of alcohol has "conditioned" the organism to a higher rate of alcohol metabolism (111, 112). The phenomenon is difficult to explain in the term of our present knowledge of the mechanism of alcohol metabolism and of the nature of enzymatic processes. Some of the reliable experimental results of the dependence of alcohol metabolic rate on alcohol concentration are assembled in table 1. It is seen here that they differ widely from each other, not only from investigator to investigator but

TABLE 1

SPECIES	AUTHOR AND REFERENCE	METHOD OF INVESTIGATION	DEPENDENCE OF ALCOHOL METABOLIC RATE ON ALCOHOL CONCENTRATION
Dog	Mellanby (105)	alcoholaemic curve	no
	Widmark (149)	alcoholaemic curve	no
	Newman and Lehman (118)	alcoholaemic curve	no
	Ewing (47)	alcoholaemic curve	yes, hyperbolic curve
	Cutting, Newman & Lee (32)	alcoholaemic curve + infusion experiments	yes, rectilinear decline, of alcoholaemic curve, but "conditioning"
	Loomis (100)	Same	no
Cat	Newman and Lehman (118)	alcoholaemic curve	no
	Eggleton (46)	alcoholaemic curve +infusion experiments	yes, hyperbolic curve
Mice	Nicloux (123)	analysis of total animal	yes, hyperbolic curve
Rat	Le Breton (91)	analysis of total animal	no
Guinea pig	Le Breton (91)	analysis of total animal	yes, hyperbolic curve
Rabbit	Newman and Lehman (118)	alcoholaemic curve	no
	Le Breton (89, 91)	analysis of total animal	yes, hyperbolic curve

Results of some experiments on the dependence of alcohol metabolic rate on alcohol concentration in the tissues. All these experiments seem to have been performed with equally reliable techniques.

in some cases investigators from the same group have obtained different results from time to time. These contradictions have naturally inspired some investigators to vary their experimental technique in order to check their findings. The most commonly used variation in the technique has been to infuse alcohol diluted with saline slowly at a constant rate for hours in an experimental animal or a person. If the infusion rate exceeds the metabolic rate, an increased concentration of alcohol in the blood will result and *vice versa*. Such experiments have been made with dogs by *Newman* and his group (111). Here it was found that a higher infusion rate was necessary to maintain a high alcohol concentration in the blood than to maintain a low level. *Eggleton* (46) obtained the same results in cats. The experiments were repeated by *Loomis* (100) in dogs, but he found that the same infusion rate (within ± 30%) was able to keep the blood alcohol level constant for several hours independently of the concentration of alcohol in the blood. All three series of experiments seem to be beyond criticism, and the only conclusion which can be drawn is that the conditions of alcohol metabolism can vary from time to time, even in the same species.

As to man, until recently experiments on several hundred persons had invariably shown no signs of a dependence of the alcohol metabolic rate on the concentration of alcohol, either in form of an asymptotic approach of the alcoholaemic curve to the zero line or a steeper slope in persons with an initial high alcohol concentration in the blood. From a practical point of view it seems well established that the human organism metabolizes alcohol at a constant rate independent of the concentration of alcohol. For low concentrations of alcohol this is confirmed by *Newman and Cutting* (115) who in experiments with man were able to maintain the blood alcohol concentration at a constant level with the same amount of alcohol slowly infused intravenously, whatever the initial blood alcohol concentration between 15 and 94 mg %. Yet even here some recent experiments show a dependence of alcohol metabolic rate on alcohol concentration. *Hjelt* (74) working for the Finnish government and *Goldberg* (54) working for the Swedish government both found a statistically significant increase of the β-factor with increased amounts of alcohol ingested. In *Goldberg's* material the β-factor was found increased about 40% when 1.5 grams alcohol per kg body weight was taken instead of 0.5 gram per kg. In both series of experiments the results were widely scattered, indicating that many experiments must be made in order to show the effect.

A quite different approach to the problem indicates that, at least in man and rabbits, the metabolic rate of alcohol is not completely independent of the concentration of alcohol. As mentioned later, tetraethylthiuramdisulphide (antabuse) is able to inhibit the second step in alcohol metabolism, the oxidation of acetaldehyde to acetate. Organisms pretreated with this substance will therefore accumulate acetaldehyde after the administration of alcohol and the concentration of acetaldehyde will depend on the rate at which acetaldehyde is formed, *i.e.*, the metabolic rate of the first step of alcohol oxidation. In rabbits (68) and in man (128) it is found that the concentration of acetaldehyde, both in untreated subjects and after antabuse and alcohol, is higher with higher concentration of blood alcohol and *vice versa*. This suggests that the oxidation of alcohol to acetaldehyde proceeds at a higher rate with higher concentrations of alcohol. On the other hand, the metabolic rate of alcohol can be calculated from the concentration of acetaldehyde in the blood, and an elimination curve in the rabbit constructed from these calculations shows so little deviation from the straight line that it lies within the analytical error of the alcohol determinations (77). We have no information about the relation between acetaldehyde production and the blood acetaldehyde concentration in man, and it is at present impossible to make a similar calculation for the human organism; but, if the conditions are similar to those in rabbits, it will also here be impossible to distinguish between a rectilinear decline and the expected slightly curved decline in the blood alcohol.

Our present knowledge can probably be summarized in the following statement: The rate of oxidation of ethyl alcohol in the organism is somewhat increased with increasing concentrations of alcohol. Within the concentrations possible in the living organism this increase is so small that the elimination curve of alcohol generally follows a straight line, and for all practical forensic purposes no error is made if we assume a rectilinear elimination curve for alcohol. Under certain conditions which have not yet been defined the dependence of alcohol metabolism on alcohol concentration may be so pronounced that the blood alcohol curve deviates significantly from the straight line. An attempt to explain this is given in a later section.

Individual Variations: Widmark concluded from his experiments that the capacity to metabolize alcohol was subject to relatively little variation within the species, and to still less variation from time to time within the same individual. Taken as a whole, later investigations have confirmed his point of view, but in some experiments a slight, but statistically significant variation in β is observed in the same individual, even when measured under apparently the same conditions (135, 136). The observed variation is, however, not very high. In man it is not found to exceed 25% in any published experiment, which is so close to the exactitude with which β can be determined that many experiments are necessary in order to demonstrate this variation with statistical significance. In man all the experiments on the variation of the alcohol metabolic rate in the same individual have been made at relatively short intervals, and no experimental evidence is found in the literature as to the order of magnitude of the variations occurring during the whole life cycle. In rats, it is shown that young animals have a much higher metabolic capacity for alcohol per unit of weight than older rats, while the capacity in chickens is the same at all ages (*cit.* after 35). The problem of the variation within the single individual may have some forensic importance, but theoretically it must be considered a minor one.

The question of whether the continued use of alcohol can increase the capacity of the organism to metabolize alcohol has been the aim of extensive investigations and discussions in earlier papers. Comprehensive reviews are given by *Bernhard and Goldberg* (12) and *Carpenter* (21). The general opinion now is that the metabolic rate of alcohol is not increased, even after long and continued use of alcohol, either in rabbits (80), dogs (114), rats (4), cats (46) or man (12, 135).

Effect of Body Temperature: Like all other chemical processes, the metabolic rate of alcohol depends on temperature. In experiments with frogs *Nicloux* (123) found that the rate was doubled for each 10° rise in temperature. *Dybing* (45) showed that β in rats with a body temperature of 20–25° was about a fourth of that found in rats with a body temperature of 38–39°. In rabbits *Fuhrman* (52) found an increase of 56% with an increase of body temperature from 25° to 37°. The variation of body temperature must, however, be rather high if any influence on the metabolic rate is to be demonstrated, and it has not been observed by all investigators. Thus *Le Breton* (94) could not demonstrate any effect of temperature in rats and *Ewing* (47) failed to show any difference in dogs after the temperature had been raised about 4° C. by means of short-wave radiothermy. In man *Fleming and Reynolds* (51) observed a slight, though not quite convincing, increase after diathermy and in a couple of experiments *Danopoulos* (34) was able to demonstrate a higher rate of disappearance of alcohol from blood in patients during attacks of malaria than in the fever-free periods, but the difference was not very high. The effect of fever induced by drugs, such as α-dinitrophenol, will be discussed later.

Influence of Metabolic Processes: From species to species there seems to be a rough relation between basal metabolism and capacity to metabolize alcohol (95); small animals with high metabolism per kg body weight have a higher metabolic rate of alcohol per kg body weight than bigger animals, but so far only mice, rats, guinea pigs and rabbits have been examined. Within the single individual, it is well established that the metabolic rate of alcohol does not follow the variations of the general metabolism. Even heavy muscular work sufficient to increase metabolism maximally is not able to increase the rate of disappearance of alcohol in the blood more than can be explained by the increased evaporation through perspiration and respiration (24, 91, 124). Less clear is the effect of increased metabolism caused by hyperfunction of the *thyroid gland* or by intake of *thyroid hormone.* Some have found an increase, some a decrease, and still others no effect (39, 70, 80, 152). *Starvation* decreases the metabolic rate of alcohol to $\frac{3}{4}$–$\frac{2}{3}$, and tissues from starving animals oxidize alcohol more slowly than normal tissues *in vitro* (97). The metabolism of fat seems to have no influence (40). The same is the case with protein metabolism. The possible influence of alanine metabolism (90) seems to be connected with the deamination of alanine to pyruvate, as will be mentioned later.

The *influence of carbohydrate* metabolism has been much discussed. *Dontcheff* (40) and *Berg, Stotz and Westerfeld* (10) showed that animals kept on a high carbohydrate diet, and consequently with a high metabolism of carbohydrate, had a higher metabolic rate than animals kept on a diet low in carbohydrates. *Dontcheff* was even able by means of measurements of the RQ to show that the metabolic rate of alcohol closely followed the amount of carbohydrate oxidized in rats (41). In mice, the alcohol metabolism is retarded after starving, but rapidly brought back to normal range for this species after a single dose of glucose. In organisms with sufficient carbohydrate reserves, the additional administration of carbohydrate is without influence on the alcohol metabolism and the rate of alcohol metabolism is found to be independent of the level of the blood sugar (27, 75, 85, 100). Only *Carpenter and Lee* (22, 23) found an increase after glucose, but their technique of determining the rate of alcohol metabolism-disappearance of alcohol from the expired air is probably less reliable than the direct measurement of blood alcohol.

A long series of investigators have *examined the role of insulin in alcohol metabolism*. Depancreatized dogs and cats show a rate of alcohol metabolism only $\frac{1}{3}$–$\frac{1}{4}$ of that of normal animals. The effect of pancreatectomy on the alcohol metabolic rate starts about 24–48 hours after the operation and is first complete 72 hours after. *In vitro*, livers from pancreatectomized cats showed only a slight capacity to oxidize alcohol, about $\frac{1}{6}$ of that of livers from similar animals treated with insulin (30). Diabetic patients seem, however, to have the same capacity to metabolize alcohol as normal persons. The effect of pancreatectomy has not been observed by all investigators, (106) and is in any case not so intense as after hepatectomy (30).

The effect of the administration of insulin to normal organisms on alcohol metabolism has been the subject of numerous investigations. Some of the results are difficult to evaluate: in many cases it is impossible to calculate the β-factor from the experimental data, and where the authors draw their conclusions from a lower peak of the blood alcohol curve after insulin and oral administration of alcohol, the effect might have been due to a delayed absorption of alcohol, and not to an effect on alcohol metabolism in itself. But even the results of experiments made with a reliable technique differ from author to author. One group find a definite acceleration of alcohol metabolism in dogs after insulin. This was first shown by *Supniewsky* (139) in 1926 and later confirmed by *Widmark* (151), *Clark and coworkers* (28–30), *Greenberg* (58) and *Newman and Cutting* (117). About 1 unit of insulin per kg body weight is necessary to obtain an effect. In rats no effect could be seen with doses of insulin up to 15 units per kg; in rats kept on high carbohydrate diet, 40 and 500 units insulin per kg body weight caused a marked increase of the alcohol metabolic rate, especially when the insulin was given during several days (42). Some investigators claim to have observed an effect in man after single doses of 20, 40 or more units of insulin (137). The maximal increase of β after insulin in experiments with man or dogs has, however, never exceeded 50%.

On the other hand, not a few investigators have found no effect of insulin on alcohol metabolism, even with high doses of insulin and the addition of extra glucose (59, 70, 100). There can thus be little doubt that the effect of insulin in this respect does not occur under all circumstances. Perhaps *Widmark's* (152) observation that he only saw the effect of insulin in dogs with a low alcohol metabolic rate and not in animals with a high metabolic rate, gives a hint of a possible explanation.

The effect of alcohol on carbohydrate metabolism is very little known. On the whole alcohol seems to have no effect on the normal or increased blood sugar. It has been claimed that the continuous intake of alcohol impairs glycogen formation in the liver, and the insulin-glucose treatment of chronic alcoholics is based on this conception. But the conditions in chronic clinical alcoholism involve a long series of other factors which have nothing directly to do with the metabolism of alcohol in these patients. All experiments on the connection between alcohol and carbohydrate metabolism have been rather primitive, as none of the experiments has given information of the fate of the carbohydrate administered or present in the organism, or about the oxidation, mobilization and formation of glycogen in the liver or the muscles, and it is difficult to suggest any explanation of the effect until further details about the carbohydrate metabolism in such experiments are available.

The experimental evidence puts it beyond doubt that there is some connection between carbohydrate and alcohol metabolism. As it is very unlikely that alcohol is metabolized by the same systems of enzymes and regulated by the same systems of hormones as carbohydrate metabolism, the hypothesis has been advanced that certain metabolites of carbohydrate are able to react with alcohol in the tissues, and here interest has been concentrated on pyruvic acid, which seems to have an effect like insulin on the alcohol metabolism. *Leloir and Muñoz* (97) observed an acceleration of alcohol oxidation by pyruvic acid and by oxalacetate in *in-vitro* experiments with liver slices. In experiments with dogs *in vivo*, *Westerfeld, Stotz and Berg* (143) found an average increase of the alcohol metabolic rate of 2–3 times after about 0.5 g to 1 g sodium pyruvate per kg body weight, and this finding is confirmed by other authors (58). An increased formation of acetaldehyde is also found after administration of pyruvate and alcohol (144). The effect of pyruvate does not seem to be found under all circumstances: *Hulpieu et al.* found no accelerating effect in dogs (75), neither could *Gregory and his coworkers* (60). The discrepancy between the results obtained in the same experimental animals with apparently reliable experimental methods is possibly due to the fact that *Westerfeld et al.* worked with animals with a much smaller initial metabolism of alcohol than *Hulpieu and coworkers*. There may be an analogy with the above mentioned observations of *Widmark*, who only found an accelerating effect after insulin in animals with a relatively slow initial metabolism of alcohol. *Bartlett* and *Barnet* found that pyruvate not only did not stimulate alcohol metabolism, but even inhibited it, both *in vivo* and *in vitro* (8). Their experimental technique differed, however, considerably from that of the other workers: Alcohol with C^{14} was given to rats, and the rate of alcohol metabolism was measured from the amount of radioactive CO_2 excreted per hour. Pyruvate delayed alcohol metabolism measured in this way considerably. A possible explanation of this will be discussed in a later section.

The accelerating effect of *dl-alanine* was first suggested by *Widmark* (148, 150), confirmed by *Le Breton* in rabbits (90), by *Eggleton* in cats (46), and most recently also found by *Westerfeld et al.* in dogs (143). The effect is of the same order of magnitude as the effect of pyruvate. *Westerfeld and his coworkers* showed an increase of pyruvate and lactate in the blood after the injection of *dl*-alanine, and as the maximal effect on alcohol metabolism in some experiments was found delayed by one hour or more, in comparison with the effect of corresponding amounts of pyruvate, they concluded that the effect of alanine is merely due to pyruvate formed in the organism from alanine by deamination (143).

Vitamin Deficiencies: In spite of the important role vitamins are known to play in general metabolism, very few investigations have been made on alcohol metabolism during vitamin deficiencies. This is the more astonishing as it is known that at least two members of the vitamin B group are found in coenzymes necessary for the metabolism of alcohol and its metabolites in the tissues: nicotinic acid which is part of the diphosphopyridine nucleotide molecule, and riboflavin which is part of the flavoprotein.

In experiments with dogs and pigeons deficient in thiamine no effect on the metabolic rate of alcohol was seen (10). This conforms well to the fact that no enzyme containing thiamine is found to play a part in any known system in alcohol oxidation; the thiamine deficiencies observed in chronic alcoholism must probably be attributed to lack of thiamine from improper food intake.

Guinea pigs fed on a scorbutogenic diet showed an alcohol metabolism about half the rate than seen in normal control animals (79). The role of vitamin C in alcohol metabolism is unknown.

Effect of Drugs on Alcohol Metabolism: Of the many drugs so far examined only dinitrophenol and dinitrocresol have been found to *accelerate* alcohol metabolism. In dogs an increase of 100–200% was found in the first experiments with α-dinitrophenol or dinitro-o-cresol (70, 15). Such large effects were not found by other investigators who claimed that the increased alcohol elimination after dinitrophenol seen in their experiments could be explained by increased alcohol excretion due to increased respiration and perspiration caused by the increased general metabolism after ingestion of α-dinitrophenol (120). *Ewing* (47) found a definite but more moderate increase of alcohol metabolism in dogs after α-dinitrophenol, even when the amount of alcohol excreted in the urine, the expired air and the sweat was taken into consideration. However, very high and toxic doses of α-dinitrophenol are necessary to demonstrate the effect. The use of α-dinitrophenol to accelerate alcohol metabolism in intoxicated persons would therefore be too dangerous for practical use in patients. The addition of dinitrophenol to the blood of artificially perfused isolated livers has no effect on the rate of alcohol disappearance from the circulating blood (48); but, *in vitro,* dinitrophenol in a concentration of 1:5,000,000 has been found to increase alcohol oxidation in liver slices by 5–10%; higher concentrations gave a definite inhibition (121).

Other drugs seem to inhibit the alcohol metabolism. In dogs, *Clark and coworkers* found that phloridzine inhibited alcohol metabolism by about 25%. They interpret this effect as due to fatty infiltration of the livers and starvation of the animals (30).

Cyanamide and antabuse, two drugs known to have a strong inhibitory effect on the second step of alcohol oxidation (acetaldehyde to acetic acid) have also a definite effect on the first step, although in doses much higher than those necessary to influence the acetaldehyde oxidation. *Gärtner* (53) found an almost complete inhibition of alcohol metabolism after the administration of cyanamide to rabbits. Also in rabbits, higher doses of antabuse (tetraethylthiuramdisulphide) result in a decrease of β to almost zero (66). A similar trend was seen in dogs (119). Smaller doses of antabuse, fully capable of inhibiting the acetaldehyde oxidation, have no effect on the rate of alcohol metabolism, either in man (65), rabbits (66, 89) or dogs (100, 113, 119).

From a practical point of view it is important whether or not it is possible to accelerate the alcohol metabolism in a person under the influence of alcohol. It will be understood that although some procedures seem to have an effect in some cases, their effect has been disputed and seems only to appear under certain conditions the nature of which has not yet been cleared up. Moreover, very few reliable experiments have been made on man. As mentioned the use of dinitrophenol is too dangerous. With insulin it is of no use to give a dose of less than 0.5 unit per kg body weight; with, for example, 40 units of insulin + glucose the effect presumably does not exceed 50%, *i.e.*, the elimination time of a certain concentration of alcohol is shortened by one third, but many patients will most likely not show any measurable effect even after this high dose.

THE SITES OF ALCOHOL METABOLISM IN THE ORGANISM

The *liver* is the most important organ capable of oxidizing alcohol. Liver slices and liver brei can oxidize alcohol *in vitro* (8, 9, 30, 97, 103), and liver forms the raw material for the isolation of alcohol-oxidizing enzymes (18, 19). More important is the fact that surviving livers, artificially perfused with blood containing alcohol, remove the alcohol from the blood at a rate corresponding to ½ to ⅝ of the rate expected in the whole organism. Such experiments have been made in dogs (48), cats (102) and rabbits (68). The earliest of these published experiments were made by *Fiessinger et al.* in 1936 (48), but the most comprehensive are the experiments of *Lundsgaard* (102) who followed not only the disappearance of alcohol but also the oxygen consumption and carbon dioxide production. He showed that the alcohol is only partially oxidized in the liver under these circumstances, presumably to acetic acid.

Some experiments seem to indicate that *muscles* can oxidize alcohol to a certain degree. *Fleischman* (50) found a lowering of the respiratory quotient when frog muscles were made to contract in a Ringer's solution containing alcohol. He also found a formation of aldehyde or ketone in muscle brei suspended in a Ringer's solution during 24 hours. He claimed that this was due to acetaldehyde. *Lehman* showed that horse muscle and alcohol are able to decolorize methylene blue in *Thunberg* experiments (96). Earlier *Hammill* (69) and *Fischer* (49) found the isolated heart capable of utilizing alcohol. Their experiments were extensive, but as they were made in 1910 and 1917, respectively, they ought to be repeated with more modern techniques. Nevertheless they fit well with the recent observation that rat diaphragm and rat heart muscle *in vitro* from $C^{14}O_2$ from $C_2^{14}H_5OH$ (8). It has already been mentioned that muscular work has no influence on the rate of alcohol metabolism in mammals.

Some investigators have detected the oxidation of alcohol by *kidney* tissue *in vitro*. In some experiments the effect was small (97), but in single paper reports an effect even exceeding that of liver tissue (8); but the role of the kidneys in alcohol metabolism in the intact organism cannot be very important.

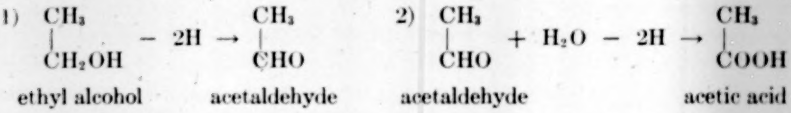
It has been discussed whether or not *brain tissue* is capable of oxidizing and utilizing alcohol. *Himwich et al.* (72) have found that the respiratory quotient of cortex tissue of rats was 0.8–0.9 after intraperitoneal injections of alcohol as against 1.0 in untreated animals. They interpret this finding as evidence for the ability of cerebral cells to oxidize alcohol, as the respiratory quotient for alcohol (0.67) is much lower than the respiratory quotient for carbohydrate (1.00) which is the normal substrate for oxidation in the brain. No perfusion experiments have been made and the results of the *in-vitro* experiments vary considerably. *Dewan* (37) found ox brain *in vitro* capable of forming acetaldehyde and acetic acid from added alcohol and chemically identified both metabolites. The oxidation requires a nicotinic acid-containing catalyst, diphosphopyridine nucleotide (see later). He also found brains from dog, cat, pig, cow, guinea pig and rabbit (all the species examined) able to oxidize alcohol *in vitro* (36). In experiments with radioactive alcohol (C^{14}) *Bartlett and Barnet* found no formation of $C^{14}O_2$ by brain tissue from rats (8). As to the significance of the possible capacity of the brain tissue to metabolize alcohol, all investigators agree that it plays a very small role in the alcohol metabolism of the total organism, and that its significance as an energy spender in brain metabolism is of no practical importance. It has been suggested that the alcohol oxidases present in the brain may have a function as a possible "detoxicator" of alcohol, and thereby as a natural protector of the brain tissue against alcohol. However, until now there is no experimental evidence for this hypothesis.

As to the other tissues *Leloir and Muñoz* have examined *testes*, *spleen* and the *intestinal tract in vitro* and did not find any oxidizing effect of these tissues (99). For the sake of completeness it must be mentioned that *Lehman* (96) found that tissue from a sarcoma catalyzed the reaction of alcohol with methylene blue in *Thunberg* experiments.

Up to the end of the 1930s there was much discussion about which organ should be regarded as the *principal* one in alcohol metabolism (see the reviews by *Kochmann* (87), *Rosemann* (133) and *M. Schmidt* (135)). Now there can be little doubt that by far the largest part of alcohol metabolism takes place in the liver, and that the tissues outside the liver play only an insignificant role. As already mentioned, artificially perfused livers have shown a capacity to metabolize alcohol corresponding to a great part of what can be expected of the whole organism. More important is the fact that eviscerated animals metabolize alcohol very slowly (28, 29). In hepatectomized dogs *Loomis* infused alcohol at the rate of 3 mg per min. per kg. This resulted in progressively increasing blood alcohol levels, although the same amount infused in control dogs gave no increase in blood alcohol levels (100). The increase of blood alcohol in *Loomis'* experiments after 2 hours infusion was of an order of magnitude which indicates that very little alcohol, if any at all, had been metabolized in the hepatectomized dogs. In partly hepatectomized animals the metabolic rate of alcohol decreased as more liver tissue was removed, to almost nil when the animals were totally hepatectomized (107, 108). These and similar results conform well with the fact that liver damage caused by arsenic, chloroform, phosphorus (125) and antimony (126) results in a lowered capacity of the organism to oxidize alcohol (4, 13, 26, 108). It is also shown that the alcohol metabolism is decreased in patients with liver cirrhosis (13). In rats *Lolli and Rubin* found that the alcohol metabolism had already fallen to $\frac{3}{5}$ of the normal value 24 hours after ligation of the bile duct (99). The variation from individual to individual is, however, too high and the effect too uncertain to allow the metabolic rate of alcohol to be used as a clinical liver function test (13).

THE INTERMEDIARY PRODUCTS IN ALCOHOL METABOLISM

There is little doubt that the oxidation of alcohol takes place in steps, and the following steps are by far the most likely:



$$1)\ \begin{matrix} CH_3 \\ | \\ CH_2OH \end{matrix} - 2H \rightarrow \begin{matrix} CH_3 \\ | \\ CHO \end{matrix} \qquad 2)\ \begin{matrix} CH_3 \\ | \\ CHO \end{matrix} + H_2O - 2H \rightarrow \begin{matrix} CH_3 \\ | \\ COOH \end{matrix}$$

Acetaldehyde: There is much essential evidence that *acetyldehyde* is an intermediate in alcohol oxidation. In *in-vitro* experiments with tissue slices or with tissue brei, acetaldehyde can be isolated as a reaction product (103). It will later be shown that with all purified enzyme systems acetaldehyde appears as the primary oxidation product of alcohol. *In vivo*, acetaldehyde is found in the blood during alcohol metabolism, and when alcohol oxidation is accelerated by means of insulin or pyruvic acid, the concentration of acetaldehyde is raised (139, 144). Acetaldehyde does not seem to be an intermediate product of normal metabolism (76). Thus the presence of acetaldehyde is specifically linked with alcohol metabolism, and a description of acetaldehyde oxidation clearly falls within the scope of this review.

The liver is the principal site for the oxidation of acetaldehyde. Evisceration of cats delays the elimination of intravenously infused acetaldehyde 4–5 times (101), and in experiments with artificially perfused rabbit livers *Hald, Jacobsen and Larsen* (68) have found such livers able to metabolize about $\frac{3}{4}$–$\frac{4}{5}$ of the amount which would be expected to be metabolized by intact animals. Other tissues, especially muscles, are not completely unable to oxidize acetaldehyde but their action is much less than that of the liver, both per gram tissue and in the whole organism. In contrast to what is generally found with alcohol, the metabolic rate of acetaldehyde depends on the concentration of acetaldehyde in the tissues. In perfused rabbit livers an acetaldehyde concentration of 7–8 mg per 100 gram is necessary to get the maximal metabolic rate of this substance, while a concentration of 1–2 mg per 100 gram only results in a metabolic rate of about half of the maximum (68).

Acetyldehyde is very readily oxidized in the organism. *Lubin and Westerfeld* (104) found that 100 mg/kg was eliminated during 10–15 min. by a cat. *Hald* *and Larsen* (64) found that rabbits could eliminate at least 3 mg per kg per min. and sometimes more for hours; in experiments with man, a person weighing about 70 kg was easily able to eliminate about 8 mg of acetaldehyde per minute, but the maximum rate is most probably considerably higher (6). From these figures it is seen that the oxidation rate of acetaldehyde to acetic acid is much faster than the oxidation rate of alcohol to acetic acid, and therefore the limiting factor is the first step, the oxidation of alcohol to acetaldehyde. Even a considerable inhibition of acetaldehyde oxidation has no influence on the rate of alcohol disappearance.

Inhibition of the oxidation of acetaldehyde: Certain drugs are able to inhibit the oxidation of acetaldehyde. Of those the effect of tetraethylthiuramdisulphide (antabuse) has been most studied. When rabbits premedicated with about 1 gram of antabuse per kg body weight are infused continuously with acetaldehyde, the concentration of acetaldehyde in the blood is considerably higher than in control rabbits infused with acetaldehyde at the same rate (64). Similar results have also been obtained by *Newman* (113) in experiments with dogs. The increase depends on the concentration of acetaldehyde and is the less pronounced the higher the infusion rate. At small infusion rates (*e.g.*, 1 mg per min.), the increase is several 100%, and near the maximum capacity of the organism to metabolize acetaldehyde the effect of antabuse is nil (64). The same can be seen in experiments with artificially perfused livers and to a less pronounced degree with artificially perfused hindlimbs (68). Antabuse interferes very little with the maximum capacity of an organism to metabolize acetaldehyde, but it increases the tissue concentration of acetaldehyde necessary to metabolize a certain amount of acetaldehyde per unit of time. For example, it may be mentioned that while livers from normal untreated rabbits require about 0.2–0.5 mg acetaldehyde per 100 gram in order to metabolize about 3 mg acetaldehyde per minute per 100 gram of liver, livers of rabbits treated with antabuse require 2–3 mg per 100 gram in order to metabolize the same amount. With concentrations of 15–20 mg per 100 gram, livers from both treated and untreated animals were able to metabolize the same maximum amount of acetaldehyde, 9–10 mg per minute per 100 gram of liver tissue (68). This fits in very well with the findings of the enzyme chemists.

Formation of acetaldehyde during alcohol metabolism: Acetaldehyde has repeatedly been found in the blood during alcohol metabolism in dogs (113), cats, rabbits (89) and man (63). As already mentioned the concentration of acetaldehyde obtained depends somewhat on the concentration of alcohol in the blood, and the theoretical consequences of this phenomenon have already been discussed. The concentration rarely exceeds about 1 mg per 100 cc with 200–300 mg of alcohol per 100 cc, and with small concentrations of alcohol only negligeable amounts of acetaldehyde are found in the blood in normal individuals. Alcohol given to animals *premedicated with antabuse* results in a much higher (2–10 times) concentration of acetaldehyde in the blood than that found with the same concentration of alcohol in normal non-treated animals (63, 66, 73, 77, 98, 104, 119). The highest concentration seen in any experiment is about

3 mg acetaldehyde per 100 cc blood. It is natural to relate this phenomenon with the effect of antabuse on acetaldehyde metabolism, and this assumption is confirmed by the close agreement between the expected concentration of acetaldehyde calculated from the rate of alcohol disappearance and the concentrations actually found (77).

Not only antabuse, but a number of other substances cause a similar increased concentration of acetaldehyde after the administration of alcohol. In experiments with rabbits this has been found to be the case with cyanamide (67), a substance which has long been known to provoke disagreeable symptoms in combination with alcohol, similar to those observed after antabuse. In hitherto unpublished experiments *Hald and Larsen* have found that tetraethylthiurammonosulphide and diethyldithiocarbamic acid also cause the accumulation of acetaldehyde. Among the analogues to tetraethylthiuramdisulphide, thiuramdisulphide has no effect, and tetramethylthiuramdisulphide has an effect somewhat higher than tetraethylthiuramdisulphide but its general toxicity is much higher. The effect declines rapidly as we pass through tetrapropylthiuramdisulphide to tetrabutylthiuramdisulphide, the effect of which is nil.

A series of other substances elicit the same clinical symptoms as cyanamide and antabuse after alcohol intake. The most frequently mentioned are some hitherto unknown substances in the fungus *Coprinus atramentarius, carbontetrachloride* and *charcoal* (109), but so far no examination has been made of the influence of these substances on acetaldehyde metabolism.

Acetic Acid: Until recently acetic acid was merely a hypothetical step in alcohol metabolism, mainly because the determination of acetic acid in tissues is rather difficult. In an ingenious way *Bernhard* has brought experimental evidence for this hypothesis since $\frac{1}{6}$–$\frac{1}{3}$ of the deuterium given with the alcohol CD_3CD_2OD was recovered as the acetate of paraacetylbenzene sulphonamide excreted in the urine and derived from simultaneously administered sulphanilamide (11). The acetate formed during the oxidation of alcohol in the liver is not exclusively oxidized in this organ, but is carried with the blood to other tissues (*e.g.*, to the muscles) and is utilized there. In *Lundsgaard's* (102) perfusion experiments with isolated livers the amount of oxygen used was too small for the total oxidation of the alcohol which disappeared. He therefore assumed that the oxidation of some of the alcohol did not proceed further than to the acetic acid stage, and his assumption was confirmed by the fact that a considerable amount of acid was found in the perfusion blood during the experiment. Under normal circumstances the acetate must be rapidly oxidized further to carbon dioxide and water. *Bartlett and Barnet* (8) showed that more than 90% of the radioactive carbon can be recovered as $C^{14}O_2$ from the expired air within 10 hours after the administration of $C_2^{14}H_5OH$. When other metabolites were given together with alcohol (*e.g.*, pyruvate), the excretion of $C^{14}O_2$ was substantially delayed. This is not in contradiction to the findings that the first step of alcohol oxidation can be accelerated with pyruvate, but is rather to be explained as a delayed oxidation of acetate formed from alcohol. Acetate is also formed as an intermediate step in normal metabolism, not only in the process of oxidation but also in the formation of fats and perhaps also glycogen, and when the general metabolic processes are increased, for instance after the administration of pyruvate, the acetate formed from alcohol will be pooled with the acetate formed from other metabolites and the oxidation thus delayed. The nutritional value of alcohol is easily explained by the fact that the second product of oxidation, acetic acid, is a compound also found during the metabolism of carbohydrate, fats and protein. The acetic acid from alcohol joins the "acetylic pool" of normal metabolism, and from this step on the metabolism of alcohol offers no special problem. The description of acetate metabolism is not within the scope of this review, and the interested reader is referred to special articles on this subject (16).

POSSIBLE BIOCHEMICAL PROCESSES OF ALCOHOL METABOLISM IN THE ANIMAL ORGANISM

In 1910 *Battelli and Stern* (9) showed that extracts from animal tissues are able to oxidize alcohol *in vitro*. In the 25 years following this basic observation practically no attempt was made to study the biochemical processes of alcohol metabolism in animal tissues, but much evidence has been gathered since the middle 1930s showing a number of possible mechanisms for the oxidation of alcohol to acetaldehyde and of acetaldehyde to acetic acid.

Alcohol → Acetaldehyde: Two widely different processes are possible as the first step of alcohol oxidation: dehydrogenation and a reaction with hydrogen peroxide. The first process is catalyzed by ***alcohol dehydrogenase***, the latter is catalyzed by ***catalase***.

Alcohol dehydrogenase: This enzyme catalyzes the transference of hydrogen from alcohol to the nicotinic acid-containing compound diphosphopyridine nucleotide, abbreviated DPN (also called cozymase in earlier nomenclature), according to the formula:

$$\text{Alcohol} + \text{DPN} \rightleftharpoons \text{Acetaldehyde} + \text{DPNH} + \text{H}^+$$

(Alcohol dehydrogenase)

The alcohol dehydrogenase from yeast was the first to be thoroughly examined It was crystallized by *Negelein and Wulff* (110) who also described it more closely. Animal alcohol dehydrogenase was studied by *Lehman* who first showed the dependence on cozymase (96). In 1938 *Lutwak-Man* purified alcohol dehydrogenase from liver and studied its properties more closely (103). About 10 years later *Bonnichsen and Wassén* crystallized the dehydrogenase from horse liver (18, 19) and this crystalline enzyme was comprehensively examined by *Theorell and Bonnichsen* (141). It differs from the yeast alcohol dehydrogenase in several respects, the most important of which are that the molecular weight of the yeast enzyme is about twice that of the liver enzyme (141) and that the yeast enzyme, unlike the liver enzyme, is inhibited by monoiodoacetate (103). Its most interesting chemical property is that it can be bound to diphosphopyridine nucleotide and to reduced diphosphopyridine nucleotide (141). The combination probably occurs between the pyridine group of the coenzyme and some sulfhydryl group in the dehydrogenase molecule. As indicated by the equation, the processes are reversible. The constants found in the experiments shows that at pH 7 in a state of equilibrium the rates alcohol: acetaldehyde will be about 60. The process nevertheless usually proceeds from left to right in the tissues in spite of the rather "unfavorable" equilibrium point, and this is naturally due to the fact that acetaldehyde is very rapidly oxidized further and thus removed from the equilibrium. The equilibrium figure is greatly increased (to 10,000) when the concentration of dehydrogenase in the system is low. The coupling of the coenzyme to the enzyme greatly favours the oxidation of ethanol to acetaldehyde, and the reaction is rather improbable without this effect.

Two of the results obtained by *Theorell and Bonnichsen* (141) are of extreme practical interest. The *turnover number* (the number of molecules of alcohol oxidized by one molecule of enzyme per minute) is found to 140 at 20°. It is probably somewhat higher at 37°, but on the other hand this figure is determined under optimal conditions which probably are not found in the organism. Using the turnover number 150, 73 grams (1 millimol, as the molecular weight of the dehydrogenase is 73,000) enzyme is able to oxidize 150×46 mg alcohol = 6.9 grams alcohol per minute, or 414 grams per hour. In order to oxidize 10 grams alcohol per hour, 1.7 grams alcohol dehydrogenase must be present in the organism. This is not an astonishingly high figure. *Bonnichsen* (18) obtained a yield of 1 gram of crystalline dehydrogenase per kg of horse liver, and thus it does not seem incredible that a human liver weighing about 1.5 kg should contain 1.5 grams of alcohol dehydrogenase, enough to oxidize the amount of alcohol actually oxidized by the human organism per hour. Another interesting point is that the *Michaëlis constant* (an expression of the affinity between enzyme and substrate) is so low that the rectilinear course of the physiological alcohol elimination (down to tissue concentrations of alcohol too low to be determined by most methods available) found in human experiments is to be expected from the kinetic data of the *in-vitro* experiments with the pure enzyme.

The alcohol dehydrogenase is not specific for ethyl alcohol, since the oxidation of propyl alcohol, amyl alcohol and butyl alcohol is also catalyzed (103, 140). Only alcohols containing the group $-\overset{|}{\underset{|}{C}}-CH_2OH$ react; secondary and tertiary alcohols do not. Very remarkable is the fact that alcohol dehydrogenase also is able to catalyze the process

$$\text{vitamin A} \rightleftharpoons \text{retinene},$$

retinene being the aldehyde corresponding to vitamin A. It has been suggested that alcohol dehydrogenase may be present in the retina and that it exerts an important physiological function in the formation of visual purple. Especially it must be emphasized that the crystalline alcohol dehydrogenase does not react with methyl alcohol (20, 141), in contrast to more impure preparations (103); the theoretical consequences of this fact will be discussed later. The reduced diphosphopyridine nucleotide transfers the hydrogen to other systems so that the rebuilt oxidized form is ready to accept hydrogen from other alcohol molecules. One of these hydrogen acceptors is the riboflavin-containing respiratory enzyme diaphorase (38) which in turn reacts with the cytochrome system and through cytochrome oxidase with free oxygen.

Leloir and Muñoz were first to demonstrate another possible acceptor for the hydrogen liberated from alcohol during its oxidation to acetaldehyde. This acceptor is pyruvic acid. The formula of the process is the following:

$$CH_3CH_2OH + CH_3CO \cdot COOH \rightarrow CH_3CHO + CH_3 \cdot CHOH \cdot COOH.$$

In vitro, the addition of pyruvate to liver slices suspended in a solution containing alcohol results in an increase of the rate of disappearance of alcohol to two to three times that in controls not containing pyruvic acid; under these circumstances lactic acid is formed (97). The experiments of *Westerfeld et al.* (143) showing the accelerating effect of pyruvic acid on alcohol metabolism in the intact organism have already been mentioned; here also an increased concentration of lactic acid in the blood of the experimental animals was found. The process has not yet been studied in detail. Probably diphosphopyridine nucleotide is essential, and naturally some enzyme or enzymes. It would be interesting to see if oxidation would proceed in a system containing pure lacticodehydrogenase, pure alcohol dehydrogenase, diphosphopyridine nucleotide, alcohol and pyruvic acid.

Catalase: A second possible mechanism for the oxidation of alcohol to acetaldehyde is given by *Keilin and Hartree* (82, 83). When ethyl alcohol and catalase are added to an oxidation system which reacts directly with molecular oxygen with the formation of H_2O_2, alcohol is oxidized to acetaldehyde with the simultaneous reduction of H_2O_2 to H_2O. The general scheme of this process is, for example, as follows: Uric acid + (uricase) + $O_2 \rightarrow$ alloxantin + H_2O_2 H_2O_2 + (catalase) + $CH_3CH_2OH \rightarrow 2\ H_2O + CH_3CHO$. Hydrogen peroxide and alcohol are bound to the catalase molecule and presumably react in this combination. The kinetics have been studied by *Chance* (25) who finds the following processes the most likely:

$$\underbrace{\text{Catalase} + H_2O_2}_{\text{(very rapid combination)}} \longrightarrow \underbrace{\text{Catalase }(H_2O_2) + C_2H_5OH}_{\text{(rapid second order reaction)}} \longrightarrow$$

$$\underbrace{\text{Catalase }(H_2O_2)\,(C_2H_5OH)}_{\text{(relatively unstable compound)}} \longrightarrow \text{Catalase} + 2H_2O + CH_3CHO$$

Not only ethanol, but also other alcohols react in a similar way; ethyl and methyl alcohol have the same rates of combination with catalase-H_2O_2, but the relative rates of combination of *n*-propanol and *n*-butanol are only 1/60 and 1/500, respectively. Liver and kidney tissues are rich in catalase and in enzymes which, *in vitro*, are able to react directly with O_2, reducing it to H_2O_2 (*e.g.*, uricase, *d*-aminoacid oxidase and tyraminase). In general, however, it must be assumed that the substrates for these enzymes are not present in the organism in concentrations high enough to explain the oxidation of 5–10 grams of alcohol per hour. The only possible exception is acetaldehyde. As will be mentioned below, acetaldehyde is oxidized by xanthine oxidase to acetic acid with the formation of H_2O_2 which in its turn oxidizes alcohol as described above. The newly formed acetaldehyde increases the supply of substrate for the primary oxidation reaction. In this way a "cyclic oxidation" of ethanol will take place. Actually this reaction occurs very readily *in vitro* in a system consisting of acetaldehyde and ethanol with highly purified xanthine oxidase and catalase. The process proceeds as follows (83):

$$\rightarrow CH_3CHO + H_2O + O_2 \rightarrow CH_3COOH + H_2O_2 \quad \text{(xanthine oxidase)}$$

$$H_2O_2 + CH_3CH_2OH \rightarrow CH_3CHO + 2H_2O \quad \text{(catalase)}$$

Acetaldehyde → Acetic Acid: A rather long series of possibilities have been mentioned in connection with the oxidation of acetaldehyde. The enzymes involved fall in two groups: the *flavoproteins* and the *dehydrogenases*. None of the known enzymes is specific for acetaldehyde; both groups catalyze the oxidation of other aldehydes, and the flavoproteins also catalyze the oxidation of cyclic compounds chemically little akin to aldehydes.

Flavoproteins: In these enzymes riboflavin is the active group that accepts hydrogen from the substrate and transfers it to free oxygen with the formation of hydrogen peroxide. The earliest known member of this group is *xanthine oxidase*, discovered in milk by *Schardinger* in 1902 and hence called the Schardinger enzyme. This enzyme catalyzes the oxidation of hypoxanthine and xanthine to uric acid, but aldehydes are also oxidized by the same enzyme. A flavoprotein with qualitatively the same properties has been isolated from pig liver, although not in a perfectly pure state (31, 55). The liver xanthine oxidase differs from the milk xanthine oxidase by having less action on aldehydes in comparison with its action on hypoxanthine and by its sensitivity to certain inhibitors. Antabuse inhibits liver xanthine oxidase but not milk xanthine oxidase (131), and liver xanthine oxidase is completely and irreversibly inactivated by 24-hours dialysis against distilled water (55).

Richert and Westerfeld have examined the capacity of tissues from several species to oxidize xanthine, and have found a high capacity in rat and mouse livers, a fairly high capacity in livers from cats, guinea pigs, cows and pigs, and little or no capacity in human livers or livers from rabbits and dogs (130). However, this does not mean that the tissues in question contain no flavoproteins able to oxidize acetaldehyde. Using the same procedure as was used for the isolation of xanthine oxidase from pig liver, *Knox* (86) has isolated a flavoprotein from rabbit liver which has no effect on xanthine; but, unlike the xanthine oxidase prepared from pig liver, it is able to oxidize cinchonidine and quinine. Besides, it has pronounced capacity to catalyze the oxidation of aldehydes. It is open to question whether or not *Knox's* enzyme can be regarded as a variant of the xanthine oxidase described above, but *Knox* (86) mentions that traces of xanthine oxidase from rabbit liver can be separated from his flavoprotein by fractionation with ammonium sulphate. No flavoprotein able to oxidize cinchonidine or quinine can be isolated from pig liver.

Dehydrogenases: In 1939 *Corran and coworkers* found that pig liver contained enzymes which oxidized aldehydes but which were not flavoproteins (31); but it was 10 years later that *Racker* first isolated from beef liver an enzyme able to react with diphosphopyridine nucleotide and acetaldehyde (129). This enzyme is called ***aldehyde dehydrogenase.*** The mechanism of reaction is as follows:

$$CH_3CHO + DPN + H_2O \rightarrow CH_3COOH + DPNH_2$$

(aldehyde dehydrogenase)

Unlike the dehydrogenation of alcohol to acetaldehyde which is catalyzed by alcohol dehydrogenase, the dehydrogenation of acetaldehyde seems to be irreversible, and all attempts to make it reversible have so far failed (129). The hydrogen of the reduced diphosphopyridine nucleotide is transferred as described above *via* diaphorase and the cytochrome system to molecular oxygen.

A reaction with other molecules of acetaldehyde seems to be possible if alcohol dehydrogenase is present in the system:

$$DPNH_2 + CH_3CHO \rightarrow DPN + CH_3CH_2OH$$

(alcohol dehydrogenase)

This means that, in the presence of appropriate enzyme systems, one of two molecules of acetaldehyde is oxidized to acetic acid, while the other is reduced to alcohol. *In-vitro* experiments demonstrating this dismutation of acetaldehyde have been described in the literature, and it has been especially studied by *Dixon and Lutwak-Man* (39). These authors claim that the dismutation of acetaldehyde is catalyzed by a single enzyme which they call ***aldehyde mutase.*** *Racker* showed that the dismutation will occur in a system with crystalline alcohol dehydrogenase, highly purified aldehyde dehydrogenase and diphosphopyridine nucleotide. His results make it possible that the dismutation of aldehyde is not the effect of a single enzyme, but a combined effect of the two enzymes. However, some discrepancies still have to be cleared up. *Lutwak-Man* found marked inhibition of the alcohol dehydrogenase by methanol, oxalate, maleate or pyrophosphate, but no effect of these substances was seen on the mutase effect, as would be expected if the combination of the two systems were responsible for the mutase effect (103). In any case not all enzymes with an oxidizing effect on alcohol or acetaldehyde can take part in the dismutation; *Dixon and Lutwak-Man* showed for instance that xanthine oxidase was ineffective in a dismutation system.

Other ways of elimination of acetaldehyde are at least theoretically possible. One way is suggested by *Stotz et al.* (138) whose views are based on the fact that acetaldehyde and pyruvic acid in tissue preparations and in the presence of diphosphothiamin react with the formation of acetoin:

$$CH_3CHO + CH_3CO \cdot COOH \rightarrow CH_3CO \cdot CHOH \cdot CH_3 + CO_2$$

It will later be shown that this reaction plays a minor role in the elimination of acetaldehyde *in vivo* (58).

Aldolase is an enzyme acting in the carbohydrate metabolism, catalyzing the process:

$$1 \text{ Mol fructose diphosphate} \rightleftharpoons 2 \text{ Mol triosephosphate.}$$

Aldolase is able to catalyze a condensation of aldehydes (including acetaldehyde) with dioxyacetonephosphate. The role played by processes of this kind is at present unknown.

Inhibition of the enzyme systems acting on acetaldehyde: Both from a theoretical and practical point of view the inhibition exerted by tetraethylthiuramdisulphide (antabuse) is important. Three series of investigations have been made on this point. *Kjeldgaard* (84) examined the effect on *Knox's* aldehyde oxidase and found that antabuse in concentrations of about 1:10,000,000 definitely inhibited the oxidation of all the aldehydes tested, including acetaldehyde. The degree of inhibition is diminished with increasing concentrations of the substrates, which suggests a competition between antabuse and aldehyde for the enzyme. This is very much in conformity with the results of the *in-vivo* experiments where the inhibiting effect of antabuse on acetaldehyde metabolism was found higher with small concentrations of acetaldehyde in the blood (64, 68). *Graham* (56) has examined the effect on *Racker's* aldehyde dehydrogenase and found an inhibitory effect in concentrations of tetraethylthiuramdisulphide of the same order of magnitude as were used by *Kjeldgaard*. *Graham* demonstrated that the inhibition can be partly abolished by increased concentrations of diphosphopyridine nucleotide, and suggests that tetraethylthiuramdisulphide also competes with diphosphopyridine nucleotide for the active centres of the enzyme. Reduced gluthathione and relatively high concentrations of ascorbic acid are able to reverse the inhibitory effect. From his experimental data *Graham* calculates the relative affinities of the enzyme protein for the substances investigated, and finds that the affinity for tetraethylthiuramdisulphide is approximately 50 times the affinity for diphosphopyridine nucleotide, and 350 times the affinity for acetaldehyde. *Richert, Vanderlinde and Westerfeld* (131) have investigated the effect of antabuse on the xanthine oxidase of livers. About 50% inhibition was found at concentrations of 1:4000 and 1:40,000 using xanthine and *p*-hydroxybenzaldehyde, respectively, as substrates. These concentrations are much higher than those found effective in *Kjeldgaard's* and *Graham's* experiments, and about as high as is necessary to inhibit a long series of other enzymes; so it seems as if the inhibition of this flavoprotein is not very specific. It is, however, interesting that *Richert and his coworkers* found that the addition of methylene blue not only increased the oxidizing effect of xanthine oxidase considerably, but also abolished the inhibition due to added antabuse. When the system was heated to 56° for 5 minutes, little effect was seen on the rate of oxidation of xanthine, but the inhibitory effect of antabuse was abolished. The authors conclude that antabuse has no effect on the dehydrogenating effect of xanthine oxidase, but that it inhibits the reoxidation of the reduced enzyme with free oxygen.

DISCUSSION

Our knowledge of the enzymatic processes involved in alcohol oxidation is still limited, and leaves many phenomena observed during alcohol metabolism in the total organism unexplained. Yet so many advances have been made in recent years in the field of enzyme chemistry that we are able to descry a connection at some points.

It has already been mentioned that the amount of alcohol dehydrogenase present in the liver is large enough to explain the elimination rate of alcohol in man, provided that the content of alcohol dehydrogenase in the human liver is of the same order of magnitude as in horse liver. The rectilinear course of the alcohol combustion curve conforms well with the kinetic data found in studies of the isolated enzyme (141). In this way the presence of alcohol dehydrogenase is fully able to explain the shape and slope of the curve showing the disappearance of alcohol after the administration of alcohol.

However, there is some indication that the dehydrogenation of ethyl alcohol by means of alcohol dehydrogenase is not the *only* pathway of alcohol metabolism *in vivo*. Methanol is presumably oxidized to formaldehyde just as ethanol is oxidized to acetaldehyde (81). The oxidation of methanol is not, however, catalyzed by pure alcohol dehydrogenase, and obviously there must be some other way of oxidation in the organism. Ethyl alcohol interferes with the enzyme systems involved in this process, as the presence of ethanol in the tissues inhibits the metabolic rate of methanol considerably (2, 8a, 153). It is to be expected that ethanol is not only fixed to the enzyme system oxidizing methanol but that it is also oxidized, and in this indirect way we may conclude that alcohol dehydrogenase is not the only enzyme active in the first step of alcohol metabolism. According to *Lutwak-Man* impure preparations of alcohol dehydrogenase are active with methanol as a substrate (103), but instead of supposing an unknown enzyme, it will be more natural to assume that the oxidation by means of catalase and hydrogen peroxide is the pathway sought for. *In vitro*, methyl alcohol is oxidized readily by this system, and moreover *Agner and Belfrage's* (2) data for the elimination rate of methanol in rabbits conform well with the kinetic data of the enzymatic processes found *in vitro* by *Chance* (25). Hence we must conclude that the catalase oxidation also plays a role in alcohol metabolism. For two reasons this form of oxidation presumably is not so important as the oxidation *via* alcohol dehydrogenase. One reason is that the kinetics of the catalase-hydrogen-peroxide process suggest a hyperboloidal decline of the alcohol curve (25). Still more convincing is that in an *in-vitro* system of catalase and H_2O_2 methanol and ethanol are oxidized at the same rate, while *in vivo* ethanol is oxidized at least 4–5 times as rapidly as methanol.

If it is supposed that the first step of alcohol oxidation is a combination of the "dehydrogenase oxidation" and the "catalase oxidation," the shape of the disappearance curve of alcohol in the organism will depend on the ratio between the effects of these two systems. If the former prevails the curve will be rectilinear with no dependence on the concentration of alcohol in the tissues. If a high proportion of alcohol oxidation is due to the catalase reaction, we should expect the rate to depend on the alcohol concentration, but this dependence would become less pronounced as the "dehydrogenase oxidation" became more predominant. On the assumption that the ratio between the two systems can vary from species to species or even under some conditions from individual to individual within the same species, it is easy to explain why some investigators have found the alcohol metabolic rate dependent on alcohol concentration, in contrast to the general opinion that such a dependence does not exist.

It is more difficult to explain the other discrepancies especially the much disputed effect of insulin and of pyruvate. This can not be done without experimental *in-vitro* data on the reaction between alcohol and carbohydrate metabolites, including pyruvate, and the kinetics of these processes. Apart from this the available concentration of diphosphopyridine nucleotide must also be taken into consideration. The turnover number of alcohol dehydrogenase depends on the concentration of coenzyme available for the formation of the enzyme-coenzyme complex. *Theorell and Bonnichsen* have expressed the opinion that, under natural conditions in the tissues, the concentration of free coenzyme can be so low that the enzyme is not fully saturated with coenzyme. In this case any process which will liberate diphosphopyridine nucleotide from other enzyme systems will result in a higher metabolic rate of alcohol. If the dehydrogenase is saturated with coenzyme the liberation of more coenzyme will naturally be without effect. It is possible that these factors might explain the conditioning of animals to a higher metabolic rate of alcohol (112) and perhaps also a part of the observed accelerating effect of carbohydrate metabolism. In any case they explain why the effect is only seen when the initial alcohol metabolic rate is low, but obviously this is merely a hypothesis which must be tested thoroughly before it can be accepted.

It is difficult to decide which of the many different possible mechanisms for the oxidation of acetaldehyde are important *in vivo*. Some of them are less probable than others. For several reasons the condensation of acetaldehyde with pyruvic acid with the formation of acetoin must be negligible. Dogs deprived of thiamin show a slightly decreased capacity to oxidize acetaldehyde (100), but no acetoin is found during alcohol metabolism (58). For the present no information is available to choose between the two types of aldehyde oxidizing enzymes, the dehydrogenase and flavoproteins. Both types are sensitive to tetraethylthiuramdisulphide, and the accumulation of acetaldehyde under the influence of this drug does not help to decide between the two possibilities. The dismutation of acetaldehyde to alcohol and acetic acid most certainly plays a minor role. *Westerfeld et al.* found no formation of alcohol in cats during the oxidation of acetaldehyde, and we have been able to confirm this in experiments with rabbits. The enzymes of acetaldehyde oxidation have not been studied as thoroughly as the enzymes of alcohol oxidation and, therefore, it is impossible for the present to give more than an outline of this second step in alcohol metabolism.

From this review it can be seen that the metabolism of few compounds has been studied so effectively as the metabolism of alcohol. Still our knowledge is deficient at several points easily found in the present review. As to the future, the reviewer's opinion is that it is a waste of time to discuss the shape and slope of alcoholaemic curves further without paying due attention to the enzymatic processes behind the elimination of alcohol. Recent progress in enzyme chemistry has made this possible, and the most important task for the future will be to fill the gap which still exists between the enzyme studies on the one hand and the *in-vivo* observations and experiments on the other. When this is done, the many riddles and discrepancies between the results of various groups of investigators will be easily explained, and we will wonder why there was ever any room for discussion.

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IONIZATION, pH AND BIOLOGICAL ACTIVITY

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1. GENERAL CONSIDERATIONS

Biological activity is known to be influenced by many physical and chemical factors; of these the degree of ionization is recognized as outstandingly significant. A change in biological response has often been observed to accompany a change in the degree of ionization of a drug, or of its biological receptor, or of both. These changes in ionization are brought about (a) by changing the pH of the medium surrounding a receptor (this may affect both receptor† and drug) or

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† *receptor:* Molecule, or part of a molecule, playing an important part in the metabolism of an organism and capable of combining with foreign molecules (see Section 6).

(b) by making a small change in the molecular structure of a drug so as to modify its tendency to ionize (4).

A high proportion of commonly used drugs are capable of ionization somewhere within the physiologically interesting pH range. Some of these, like sodium chloride, remain completely ionized within this range, but the great majority ionize to different degrees as the pH is varied.

The variable ionization of drugs would not be a matter of great interest to pharmacologists if both an ion and its non-ionized counterpart (*i.e.*, molecule) had the same biological action. However, in all cases which have been investigated, the ion and its corresponding molecule behave differently, so that the control of ionization is seen to be essential in biological experiments if they are to yield their full content of meaning. In some cases, the difference in action between an ion and its molecule partakes of an all-or-nothing character.

2. THE CHEMISTRY OF IONIZATION

Many substances are known which do not increase the electrical conductivity of water when dissolved in it. These are called non-electrolytes (chloroform and ether are examples) and they depress the freezing-point of water proportionally to their molar concentration. Acids, bases and salts, on the other hand, all increase the electrical conductivity of water when dissolved in it. These are called electrolytes, and the majority of drugs are electrolytes. All electrolytes depress the freezing-point of water to a greater extent than would have been expected from their molar concentration. In the cases of hydrochloric acid, sodium hydroxide and sodium chloride this depression is double what would have been expected and this led the Swedish chemist Arrhenius to formulate the theory of ionization of electrolytes (1884–1887), which has been brought up-to-date by Debye and others. Thus, in solution, hydrochloric acid consists entirely of hydrogen kations and chloride anions ($H^{\oplus}$ and $Cl^{\ominus}$), sodium hydroxide of sodium kations and hydroxyl anions ($Na^{\oplus}$ and $OH^{\ominus}$) and sodium chloride of sodium kations and chloride anions ($Na^{\oplus}$ and $Cl^{\ominus}$). Sodium sulphate gives three times the expected depression, and it has been shown that instead of each expected molecule of Na_2SO_4, three ions are present, namely, two sodium kations and one sulphate anion ($SO_4^{\ominus\ominus}$).

(i) *The Complete Ionization of Salts*

In general, salts are completely ionized in solution (11, 17), but there are a few exceptions, the halides of mercury, cadmium and lead being the most notable. Bi- and tri-valent metals form complexes with phosphoric acid and with polyvalent organic acids (such as citric acid or glycine), but these complexes are not true salts and have little tendency to ionize (see section 7, below).

Not only are the strongest solutions of salts completely ionized, but fused salts are also (32, 41). Moreover, X-ray analyses of a salt, for example, sodium chloride crystals, show that no *molecules* of NaCl exist, the chloride and sodium ions being evenly spaced throughout the crystal, as in solution. Thus, the old conception that salts could, under some circumstances, exist as molecules is seen to be unfounded. It is true that the ions in crystals and fused solids are not hydrated as in solution and hence are somewhat different in properties, for example, they are poorer conductors of electric current. In strong solutions (*e.g.*, above 10 per cent), there is a pronounced tendency for oppositely charged ions to cluster together as in crystals, an effect which becomes more pronounced when both ions have valencies higher than one. Thus magnesium sulphate is *dissociated* to the extent of only 50 per cent in decinormal solution, notwithstanding the fact that it is completely *ionized* at this and all other concentrations; complete dissociation is achieved on further dilution.

Because of the great dilutions which he commonly uses, it is seldom necessary for a biologist to consult tables (23, 28) of *thermodynamic activity coefficients* which have been drawn up for the conversion of *ionic concentrations* into *ionic activities* (which are the concentrations of solitary, fully hydrated ions). Apart from the exceptional halides of mercury, cadmium and lead (mentioned above), it is safe to take activities of inorganic salts as equal to concentrations at the dilutions commonly used. Organic salts may be treated in the same way, except where one of the ions is large (*i.e.*, with a formula-weight of over 200; see Section 7 (iv)).

Because salts are completely ionized, they have no biological properties other than those of the individual ions of which they are composed. Thus calcium chloride can have no conceivable physiological effects other than those peculiar to calcium ions and to chloride ions. This simple conception needs modification when a salt is derived from either a weak acid or a weak base, because more or less of the non-ionized acid or base will be liberated by hydrolysis (see (ii), below) thus adding its own biological effect to those of the constituent ions of the salt. We shall see (vi, below) how this hydrolysis can often be overcome by a small change in pH.

That the physiological properties of a salt should be no more nor less than the sum of those of its ions is often overlooked by biological workers. For example, Hata (37) examined the toxicity of 2:8-diamino-10-methylacridinium chloride and iodide for the mouse, and found the iodide half as active, weight for weight. He then compared the ability of these substances to save the lives of mice infected with streptococci; once again the iodide was half as active. Now, as both of the anions are biologically inert at the great dilutions tested, the biological activities must be proportional to the amount of kations in these substances (which are completely ionized in neutral solutions (3)). The formula-weights are respectively 260 and 351, so that the iodide should have 74 per cent of the potency of the chloride. The biological results are in as good agreement with this calculation as could reasonably be expected for serial dilutions. However, the author gave no indication that he realized that his results were inevitable, for he had only been comparing the diaminomethylacridinium kation with itself.

The antibacterial properties of a number of other acridine salts have been tested as their chlorides, sulphates, nitrates and iodides (12), and no effect attributable to the acid radical was found, beyond experimental error. This was only to be expected. However, in 1944, two authors published a statement that the hydrochloride of "Atebrin" is 32 times as bacteriostatic as the corresponding lactate against haemolytic streptococci, and in the next year they stated that the formate was four times as active as the tartrate against *Clostridium welchii*. These observations were published without the comment for which they obviously call. Now, it is conceivable that "Atebrin", because of its high formula-weight of 400, is showing thermodynamic activity effects and that these would vary with different anions. However, this could not be the explanation in the present case because far less striking differences were seen when these salts were tested on other organisms. This makes it likely that the salts which appeared to be less potent were not completely in solution in the relevant tests (undissolved material does not usually enter into rapid equilibrium with the surrounding fluid).

(ii) *The Ionization of Acids and Bases*

Unlike salts, acids and bases need not be completely ionized in solution. Strong acids (*e.g.*, hydrochloric acid) and strong bases (*e.g.*, sodium hydroxide) are completely ionized in the pH range of 0–14, but many biologically-active substances are weak acids or bases and hence show variable ionization within this pH range. Even small variations in pH on either side of the neutral point (pH 7) make considerable changes in the proportions of drug ionized in such cases as barbiturates, alkaloids, local anaesthetics and anti-histaminics. Several examples will be given later.

Salts which are formed from a weak acid or from a weak base are not stable in solution, but hydrolyse partly to the acids and bases from which they are derived. In this way a salt (which is by definition completely ionized) can give rise to an acid or base which is incompletely ionized. This situation is not as confusing as it may seem because the degree of ionization in solution depends on only two factors, the pH and the pK_a. The latter (which will be defined in (iv) below) is a constant for any acid or base. Hence, if the pH is controlled, the degree of ionization depends only on the nature of the acid (or base) added, *regardless of whether or not it has previously been neutralized*. Thus, the same ratio of atropine ions to atropine molecules will result from the addition of atropine hydrochloride, atropine sulphate or free atropine to a bath that has been buffered at pH 7. If the pH of the bath is raised, the proportion of atropine ions to atropine molecules will decrease, but the new ratio will again be independent of the form in which the atropine was added.

Not long ago, a paper was read at a scientific congress in which the author investigated the comparative effect of boric acid and one of its sodium salts (borax) on blowfly larvae. Had the substances been used as such, some physiological difference might have been observed because boric acid is acidic and borax is alkaline. However, the author had, very properly, carried out his experiments in a buffered solution with the result that no significant chemical difference existed between his two solutions. He actually found that both substances gave the same quantitative response. Although this puzzled him at the time it does show that the biological technique was sound because the experiment actually tested nothing else.

In 1944, the bacteriologists whose work with "Atebrin" has been referred to (under (i), above) published another paper in which they found that 5-aminoacridine was 64 times more bactericidal than its hydrochloride (against pneumococcus type III in glucose-broth). The authors did not say whether their medium was buffered. If it was buffered, the same result should have been obtained in both cases and hence the technique was at fault. If the medium was not buffered, the results could not have quantitative significance because of the differences in pH between solutions of a strong base and of its salts (the striking effect of pH changes on the antibacterial action of acridines has been known since 1919 (13, 31)).

(iii) *The Ionization Constant* (K_a)

An essential part of Arrhenius's theory of ionization was the application of the *law of mass action* to describe the state of ionic equilibrium. Thus, acetic acid (CH_3COOH) is a weak acid which ionizes in water to give some hydrogen ions ($H^{\oplus}$) and some acetate anions ($CH_3COO^{\ominus}$). The product of the concentration of the ions (which is $[H^{\oplus}][CH_3COO^{\ominus}]$ always bears a fixed ratio to the concentration of the unaffected molecules $[CH_3COOH]$. This ratio is called the acidic ionization constant (K_a), or more simply the ionization constant. Thus:

$$K_a = \frac{[H^+][CH_3COO^-]}{[CH_3COOH]} \qquad \text{(i)}$$

and this has been found, experimentally, to be 1.75×10^{-5} (at 25°).

Sometimes the expression "dissociation constant" is used for ionization constant, but the latter is more precise. Many complexes, such as enzyme systems, "dissociate" into their components, and the relevant equilibria can be expressed by dissociation constants similarly derived from the law of mass action. Nevertheless, such constants are not usually ionization constants.

The state of ionization of weak bases also can be described by acidic ionization constants. For example, ammonia is a weak base which can take up hydrogen ions to form ammonium ions. This is, of course, equivalent to thinking of the ammonium ion ($NH_4^{\oplus}$) as a weak acid which ionizes in water to give some hydrogen ions ($H^{\oplus}$) and some molecules of ammonia (NH_3). Thus:

$$K_a = \frac{[H^+][NH_3]}{[NH_4^+]} \qquad \text{(ii)}$$

and this has been found, experimentally, to be 5.5×10^{-8} (at 25°).

The use of acidic constants to describe the ionization of bases was introduced in 1923 by Brönsted (10) who noted the formal similarity between equations (i) and (ii). It is most advantageous to have the ionization of both bases and acids expressed on the same scale, but earlier workers wrote,

$$K_b = \frac{[OH^-][NH_4^+]}{[NH_4OH]} \qquad \text{(iii)}$$

and the value of K_b (the basic dissociation constant) was found to be 1.8×10^{-5} (at 25°). Equation (iii) is of little use because it does not touch the heart of the matter which is this: an acid produces hydrogen ions and a base receives them. Thus both acid and base can be related in terms of a single quantity, their affinity for the hydrogen ion. This relationship requires the use of the acidic ionization constant for both acids and bases.

(iv) *The Definition of pK$_a$*

Ionization constants are small and inconvenient, but their negative logarithms (known as pK_a values) are convenient to speak and to write. Thus the pK_a of acetic acid is 4.76 and of ammonia, 9.26. The older literature often gives pK_b values for bases (*e.g.*, 4.74 for ammonia); these can be converted to pK_a values by subtraction from the negative logarithm of the ionic product of water (K_W) at the temperature of determination. The value of pK_W is 14.16 at 20°, 14.00 at 25° and 13.58 at 37°.

It is evident that pK_a values provide a very convenient way of comparing the strengths of acids (or of bases). The stronger an acid is, the lower its pK_a; the stronger a base is, the higher its pK_a.

An acid or base when half ionized has a pH equal to its pK_a. When an acid is 10 per cent ionized (or a base is 90 per cent ionized), the pH is 1 unit below the pK_a. When an acid is 90 per cent ionized (or a base is 10 per cent ionized), the pH is 1 unit above the pK_a. Any acid or base is an effective buffer within the range from one unit below to one unit above the pK_a value. Biologists can therefore use initiative in selecting buffers suitable for particular experiments. The range in the literature is unnecessarily restricted; it neglects kations, and uses many anions (*e.g.*, citrate and phosphate) which form nonionized complexes with the ions of essential trace metals. Thus, phosphate buffer inhibits the action of *iso*citric dehydrogenase in the pig heart by removing manganese (42).

(v) *Tables of Useful pK$_a$ Values*

The common pK_a values may be obtained from standard textbooks (*e.g.*, 9). Others are easily located through *Chemical Abstracts* where they are indexed under, "Ionization, electrolytic." Table 1 gives the relative strengths of some common acids and bases; this table is intended to be committed to memory. Acids and bases of equivalent strength will be found opposite one another.

A value of 4-5 (*c.f.* acetic acid) is typical of a great many monocarboxylic acids, both aliphatic and aromatic. The value of 10 for phenol is typical also of its homologues (*e.g.*, thymol and the cresols). Acids with pK_a values much greater than 7 scarcely redden litmus.

The value of 11 for ethylamine is typical of aliphatic bases; that of 5 for aniline is typical of aromatic bases which are much weaker. As pK_a figures are logarithms, there is a difference of one million-fold (*i.e.*, antilog 6) between the strengths of ethylamine and aniline.

The presence of electron-attracting groups (*e.g.*, —Cl or —NO_2) weakens bases and strengthens acids, usually by 1 or 2 pK_a units; but when the group is suitably placed, the effect can be made much greater (*c.f.* aniline and *p*-nitroaniline in

Table 1; phenol and trinitrophenol (picric acid, pKa 0.8); acetic acid and trichloroacetic acid ($\mathrm{pK_a}$ 0.7)). It is also possible for the chemist to strengthen bases and weaken acids to almost any desired extent.

A selection of polybasic acids is given in Table 2. It is interesting to note how the values for citric acid lie close together, whereas those for phosphoric acid are widely spaced.

The $\mathrm{pK_a}$ values of several naturally occurring bases will be found in Table 3. The values for alkaloids were determined by Kolthoff in 1925 (40). Acetylcholine and curarine do not appear to have been investigated but probably resemble other quaternary bases (*e.g.*, tribenzylmethylammonium chloride, which is 13.4 at 18°). Guanidine ($\mathrm{pK_a}$ 13.7 at 25°) is a strong organic base of another type, being strengthened by a resonance effect which favours the ion at the expense of the molecule.

TABLE 1

Approximate strengths of some common acids and bases

ACIDS	$\mathrm{pK_a}$	BASES	$\mathrm{pK_a}$
Hydrochloric acid	*	Sodium hydroxide	*
Oxalaic acid	2		12
	3	Ethylamine	11
	4		10
Acetic acid	5	Ammonia	9
Carbonic acid	6	Quinine, Strychnine	8
	7		7
	8		6
Hydrocyanic acid, Boric acid	9	Aniline, Pyridine	5
Phenol	10		4
	11		3
	12		2
Glucose	13	*p*-Nitroaniline	1

* Too strong to have a $\mathrm{pK_a}$ value.

(vi) *Calculation of the Percentage Ionized*

The percentage ionization of any acid or base can be calculated if two things are known, (a) its $\mathrm{pK_a}$ value and (b) the pH at which the information is required. For a base, equation (iv) is used.

$$\text{Per cent ionized} = \frac{100}{1 + \text{antilog}\,(\mathrm{pH} - \mathrm{pK_a})} \tag{iv}$$

and for an acid, equation (v) is required

$$\text{Per cent ionized} = \frac{100}{1 + \text{antilog}\,(\mathrm{pK_a} - \mathrm{pH})} \tag{v}$$

These equations show that the degree of ionization varies with the pH. The relationship between ionization and pH is not linear, but sigmoid as Fig. 1 clearly shows. From this figure, it is evident that a small change in pH can make a large change in the percentage ionized, particularly if the values of $\mathrm{pK_a}$ and pH lie close together. For example, if pilocarpine (pK$_a$ = 7.3) is being used at pH 7.6 it is only 33 per cent ionized; but if used at pH 7.0, it is 67 per cent ionized.

TABLE 2

pK$_a$ *values of some polybasic acids (20°C)*

ACID	ANION	pK$_a$
Carbonic	mono-	6.46
	di-	10.35
Citric	mono-	3.06
	di-	4.74
	tri-	6.41
Oxalic	mono-	1.19
	di-	4.29
Phosphoric	mono-	2.16
	di-	7.13
	tri-	12.30
Sulphuric	mono-	*
	di-	1.93
Tartaric	mono-	3.01
	di-	4.55

* Too strong to have a pK$_a$ value.

TABLE 3

pK$_a$ *values of some naturally-occurring bases (15°–20°C)*

BASE	pK$_a$	BASE	pK$_a$
Adrenaline		Morphine	
kation	10.2	kation	8.2
anion	8.7	anion	9.9
Arecoline	7.5	Nicotine	8.2
Atropine	10.0	Papaverine	6.3
Cocaine	8.7	Pilocarpine	7.3
Codeine	8.3	Piperidine	11.6
Emetine		Quinine	
mono-kation	8.6	mono-kation	8.4
di-kation	7.7	di-kation	4.7
Eserine	8.2	Quinidine	8.9
Ethanolamine	9.5	Strychnine	8.4
Gelsemine	9.4	Tyramine	
Histamine		kation	9.3
mono-kation (NH_2)	9.9	anion	10.9
di-kation	6.1		

Thus a variation of only 0.6 unit in the pH has doubled the concentration of kations and halved the concentration of molecules. Table 4 gives an overall picture of the changes in ionization produced by variation in (pK$_a$ − pH), and

Table 5 provides a magnified picture of this effect in the critical region where $\mathrm{p}K_a$ and pH are nearly equal.

3. EXAMPLES OF SUBSTANCES WHOSE CHEMICAL REACTIVITY DEPENDS UPON THE DEGREE OF IONIZATION

The organic chemist often has to use strong acid or alkali to facilitate a synthesis. Reactions which require such reagents for their initiation have been shown to depend on the ionization of a difficultly ionizable substance (34). Dimethylaniline (I) provides an interesting example. This substance ordinarily nitrates in the *o*- and *p*-positions, as shown by the arrows. However, if sufficient concentrated sulphuric acid is present for the substance to be entirely converted to the kation (II), nitration occurs exclusively in the *m*-position. The hydrolysis of sucrose, when catalysed by dilute acids, is actually the hydrolysis of the small amount of sucronium kation which the acid continually forms (34).

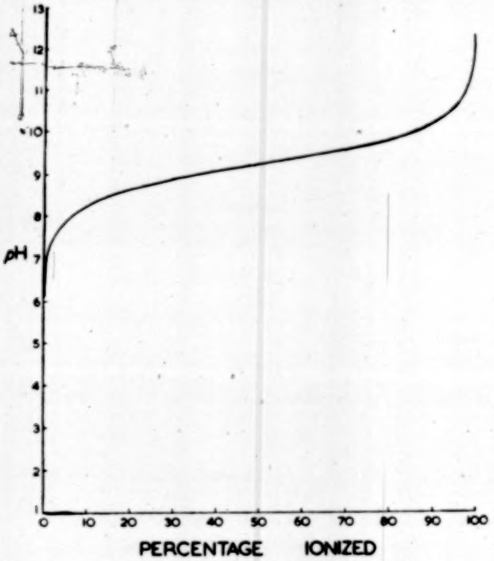
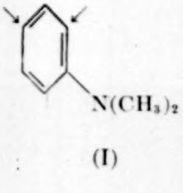
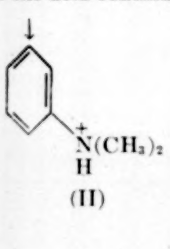


Fig. 1. Typical curve obtained in the potentiometric titration of an acid (boric acid, $\mathrm{p}K'_a$ = 9.21 at 20°).



(I)



(II)

Quite obviously the action of drugs is not concerned with ionizations occurring so far from the neutral point as these examples; a better model is supplied by ascorbic acid. This substance in alkaline solution is readily oxidized by air to give dehydroascorbic acid. Now, ascorbic acid is capable of giving three different ionic species, the ratios of which vary according to the pH, in the usual way. These three species are the di-anion, the mono-anion and the molecule. As Table 6 (top half) shows, decreasing the alkalinity slows the rate of oxidation in

TABLE 4[*]

Calculation of the extent of ionization, given pK_a *and* pH

$pK_a - pH$	PER CENT IONIZED (IF ANION)	PER CENT IONIZED (IF KATION)
−4	99.99	0.01
−3	99.90	0.10
−2	99.01	0.99
−1	90.91	9.09
0	50.00	50.00
1	9.09	90.91
2	0.99	99.01
3	0.10	99.90
4	0.01	99.99

[*] An extended form of this Table is available (4).

TABLE 5

Calculation of the extent of ionization where pK_a *is close to* pH

$pK_a - pH$	PER CENT IONIZED (IF ANION)	PER CENT IONIZED (IF KATION)
−0.9	88.81	11.19
−0.8	86.30	13.70
−0.7	83.37	16.63
−0.6	79.93	20.07
−0.5	75.97	24.03
−0.4	71.53	28.47
−0.3	66.61	33.39
−0.2	61.32	38.68
−0.1	55.73	44.27
0	50.00	50.00
+0.1	44.27	55.73
+0.2	38.68	61.32
+0.3	33.39	66.61
+0.4	28.47	71.53
+0.5	24.03	75.97
+0.6	20.07	79.93
+0.7	16.63	83.37
+0.8	13.70	86.30
+0.9	11.19	88.81

proportion as the concentration of di-anion is lowered, even though the proportions of the other two species are thereby increased. In the presence of cupric ions, the autoxidation of ascorbic acid goes much faster; but the mechanism is obviously different, for here the rate is proportional not to the concentrations of the di-anion or of the molecule, but to that of the mono-anion (Table 6, lower half).

Malonic acid is readily decarboxylated to acetic acid when in the molecular condition; however, the mono-anion decarboxylates at only one tenth this rate and the di-anion does not decarboxylate at all (33).

4. HOW IONIZATION CAN INFLUENCE BIOLOGICAL ACTION

(i) *Ionic Bonds and Covalent Bonds*

The ready reversibility of drug action by dialysis, which has so often been demonstrated, points to the rarity of covalent bonds being concerned in drug-receptor combinations, because covalent bonds require a good deal of energy for breaking. In many cases, drug and receptor appear to be held together by ionic bonds reinforced by the so-called secondary linkages (hydrogen bonds and van der Waals' bonds). Hence changes in the pH of the medium bathing a receptor, whether inside the cell or outside, would be expected, in many cases, to bring about a change in biological response (see section 6). Conversely, a change in the degree, or even the kind, of biological effect can be expected from a drug employed at a new pH, or from an analogue of the drug, differing in pK_a.

TABLE 6

Autoxidation of ascorbic acid

pH	DI-ANION %	MONO-ANION %	MOLECULE %	VELOCITY * CONSTANT MIN.$^{-1}$ × 10^3	
9.21	0.5	99.5	—	22	No metallic ions
8.71	0.2	99.8	—	11	
7.61	0.01	99.99	—	0.7	
5.80	—	97.8	2.2	0.1	
4.70	—	79.4	20.6	0.03	
9.31	0.6	99.4	—	101	Copper-catalyzed
7.49	0.01	99.99	—	170	
6.12	—	99.0	1.0	134	
5.08	—	90.1	9.9	91	
3.87	—	36.0	64.0	26	
2.59	—	2.9	97.1	2	

* Weissberger & LuValle (63).

Further, the examples given in Section 3 make it clear that each species of a substance (whether di-ion, mono-ion or molecule) has its individual chemical reactivity where the making and breaking of covalent bonds is concerned. Now the breaking of covalent bonds can bring about the degradation of a drug to a more biologically effective substance (*e.g.*, chloral hydrate to trichloroethyl alcohol pamaquine or its quinone, proguanil to its triazine) or to biologically inert material. Hence variations in pH or pK_a can influence the reactions between tissues and drugs and thus can control the nature and quantity of material to reach the receptors.

There are at least two other important ways in which ionization influences biological responses, *viz.*, adsorption at surfaces and the penetration of membranes.

(ii) *Adsorption at Surfaces*

Two main types of adsorption should be distinguished, (a) indiscriminate and (b) specific.

Indiscriminate adsorption, as shown by the common soaps, does not depend upon great affinity between the substance and the surface on which it becomes adsorbed. On the contrary, it depends mainly on the feeble ability of such substances (because of their predominantly hydrophobic nature) to unite with water molecules. If the hydrophobic "tails" are only a few carbon atoms long, they can be accommodated between the water-molecules, but molecules with a more markedly polar/non-polar character tend strongly to be squeezed out of solution by the high internal pressure of the water molecules which constantly tend to unite by hydrogen bonds with oxygen- or nitrogen-containing groups and, particularly, with other water molecules. Aqueous solutions of soaps, and of other indiscriminately adsorbable substances, are therefore squeezed out of aqueous solution, by the water molecules, on to any surface that presents itself whether it be the glass walls of the containing vessel, the air/water interface at the top of the solution or the skin of the experimenter's finger. Such deposits remain in dynamic equilibrium with the bulk and are usually only one or a few molecules thick. Ionization, usually, is unfavourable to such indiscriminate adsorption because the ion, being the more highly hydrated of the two species, has the greater tendency to remain in the water. Thus, from a dilute solution of soap, oleic acid is more strongly adsorbed than the oleate ion. The study of indiscriminate adsorption (*e.g.*, by means of surface-tension studies at the air/water interface) has gradually lost its fascination for students of drug-action in proportion as they have realized that indiscriminate adsorption is unlikely to lead to specific action.

Specific adsorption, on the other hand, is to be found among molecules that are quite well hydrated. It depends upon a complementariness of shape or charge between the drug and the receptor on which it exerts its disturbing effect. When the complementariness is one of charge, obviously the ion is more likely to be adsorbed than the molecule. The acridine antibacterials, discussed below, may be taken as examples of specific adsorption. More striking examples still are furnished by the vitamins of the B group which, perfectly water-soluble and occurring as they do in food at immense dilution, nevertheless become concentrated on their complementary surfaces, which are enzymes, with astonishing efficiency. This efficiency probably owes much to the universal phosphorylation of these substances which converts them into anions before they become coenzymes.

(iii) *Penetration of Membranes*

Molecules with only one water-attracting group readily penetrate natural membranes provided that no more than 12 carbon-atoms are present (24). If, however, the water-attracting group is ionized, this penetration is retarded because the ionic group is held by unlike charges on the outer surface of the membrane and repelled by like charges. Davson and Danielli (24) quote many instances where the ionic form of a substance has been shown to penetrate membranes very much more slowly than the corresponding neutral molecule (see also 39). This phenomenon naturally brings to mind the ease with which neutrons, but not electrons or protons, penetrate the highly charged interior of atoms. It must not be supposed, however, that no ions ever penetrate natural membranes. In the first place specific mechanisms appear to exist for biologically useful ions (the human gut permits the ready passage of sodium and chloride ions but is relatively impervious to magnesium and sulphate ions). In the second place a non-penetrating ion can often be made penetrating by the addition of a lipophilic group; the chloro- and other lipophilic groups of the antimalarials mepacrine ("Atebrin") and chloroquine may assist thus in the necessary penetration of red blood cells.

A further interesting effect of ionization upon biological action can now be considered. The action of a drug obviously depends not only upon its ability to combine with some vitally important substance in a particular kind of tissue, but it also depends upon the ability of the drug to reach that tissue. Now a drug with a pK_a between 6 and 8 is in the interesting position that, at the physiologically important pH of 7, it is always in equilibrium with at least 10 per cent of its more poorly-represented ionic species. Such a drug will come to membranes through which only the non-ionic species can pass. Yet, as soon as this species has penetrated, it is likely to encounter an aqueous medium of similar pH value, and in this it is obliged to re-form ions until the same degree of ionization exists on both sides of the membrane. Quite a large number of drugs have pK_a values between 6 and 8, including the local anaesthetics and many common alkaloids (see Section 2 (v)). Some other families of drugs have pK_a values which lie entirely outside this range and hence they have a different pattern of distribution in the body, a pattern which may help to explain their different types of action.

(iv) *The Stability Constant* (K_s) *Governing Drug-Receptor Unions*

At one time it was thought that these questions of the degree of ionization would not have the importance in biology which they are now known to have. It was believed that in proportion as the biological receptors removed the toxic ionic species, more of it would be generated from the other species by the Mass Action Law, as in equation (ii) above. This may, of course, prove to be the case in some types of drug action not yet investigated. On the other hand, many examples are already known where biological activity is highly dependent upon the degree of ionization. This indicates that the combination between drug and receptor is usually weak and completely reversible in nature.

Let us consider, for example, a system where bacteria are in equilibrium with a low concentration of an antibacterial kation and a high concentration of the corresponding molecule which is non-antibacterial. In such a system, exhaustion of the kation by the bacteria would lead to the generation of significant supplies of kation from the molecule but the bacteria are themselves constantly shedding this kation back into the solution. Each bacterial anion (represented as $A^{\ominus}$), provided it is accessible to these kations, will enter into an equilibrium with them as in equation (vi) where K_s is the stability constant describing this equilibrium.

$$K_s = \frac{[ABH]}{[BH^{\oplus}][A^{\ominus}]} \qquad \text{(vi)}$$

ABH (the drug-receptor complex) is treated as an adsorption complex; it contains ionic bonds, but is also held together by secondary valencies as well. It may be visualized as a salt with a very high thermodynamic activity coefficient (see Section 2(i)).

When K_s and K_a are of similar magnitude, it is obvious that any deficiency of antibacterial kations ($BH^{\oplus}$) in the solution will be replenished from the drug-bacterium complex (ABH) as easily as from the non-ionized drug (B). Under these conditions the degree of ionization of the drug governs its efficiency; it is not enough that ions should be present, they must be present abundantly in order to force more $A^{\ominus}$ to become ABH.

The actual situation may be governed by several K_s values, because not all the kinds of groups (on the bacterium) with which the antibacterial combines are likely to be of vital importance for the existence of the bacterium. The combinations with the non-vital groups may be quantitatively great and can lead to the wastage of sub-optimal amounts of kation (50).

5. EXAMPLES OF SUBSTANCES WHOSE BIOLOGICAL ACTION DEPENDS UPON THE DEGREE OF IONIZATION

(i) *Substances which are Least Active when Ionized*

In 1921, Vermast noticed a tendency for weak acids to exhibit their biological activity most fully at those pHs where they were not ionized (62). One of the best authenticated cases of this kind is the inhibition of cell division in echinoderm eggs by salicylic acid (57). It can be seen from the upper curve of Fig. 2 that this acid is more active at pH 5 than at any other pH tested. At pH 5, a larger proportion of the salicylic acid ($pK_a = 3.0$) is in the form of molecules than at any higher pH, *viz.*, 0.99 per cent (see Table 4). When the concentration of salicylic acid present as molecules is calculated for each inhibitory concentration of total salicyclic acid (molecules + anions), the lower curve is obtained. It is evident from this lower curve that the inhibitory concentration of salicylic acid molecules remains the same regardless of pH; the only simple explanation is that the molecules, but not the anions, of salicylic acid inhibit the division of these eggs. It would be expected that at lower pH values salicylic acid would be a more powerful inhibitor because it would contain a higher ratio of molecules co anions and one wonders why this region was not explored. However, complications may occur. Firstly the organism may not show the indifference to pH change that it apparently does throughout the experimental range. Secondly the receptor for salicylic acid may undergo a change in ionization as the pH falls and would then hardly be likely to have the same affinity as before (see section 6).

Similarly, it has been found (19) that all members of a series of 30 barbiturates enter both eggs and larvae of the sea-urchin Arbacia ***exclusively as molecules.*** Moreover, the resulting depressions of cell division and of respiration were shown to be entirely due to molecules.

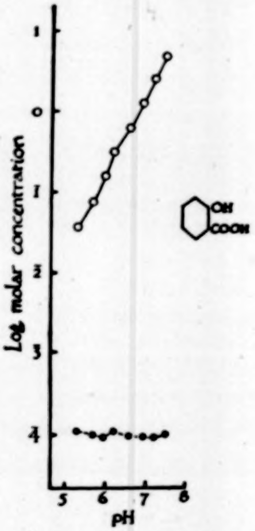


FIG. 2. The effect of pH on the concentrations of salicylic acid required to stop the cell division of *Echinarachnius parva*. Upper curve: total drug (= ions + molecules). Lower curve: molecules.

The action of *p*-aminobenzoic acid on the protozoon ***Polytomella caeca*** is of this kind. *p*-Aminobenzoic acid has a basic pK_a value of 2.65 (below this, it is largely kation) and an acidic pK_a value of 4.82 (above which it is largely anion). The maximal percentage of molecules occurs roughly at a pH equal to the average of these two figures, *viz.*, at pH 3.73. At this "isoelectric point", about 95 per cent of the acid is present in forms which are neither anions nor kations, *viz.*, about 85 per cent molecules and 10 per cent zwitterions (the latter are an ionic species to be discussed in Section 7 (ii)). It is interesting to see from Fig. 3 that the reversing action of *p*-aminobenzoic acid against sulphanilamide is greatest at this pH (43). For some other organisms (*E. coli, Aspergillus niger*), the anti-sulphanilamide action of *p*-aminobenzoic acid varies little with pH which suggests that the anion and the molecule may have comparable effects in these cases (43).

(ii) *Substances which are Most Active when Ionized*

It is now known that many thousands of kinds of organic kations are antibacterial (1), but this knowledge does not extend back very far. In 1912, Morgan and Cooper (47) found that aliphatic amines (which exist mainly as kations at pH 7) were bactericidal and aromatic amines (which exist mainly as molecules at pH 7) were not. However, these authors did not sense the connexion between antisepsis and organic kations for they stated that antibacterial activity was due "to the presence of hydroxyl ions liberated through ionization of the alkylammonium hydroxides which are formed through the combination of the amines with water." In other words, they ascribed the effect to alkalinity. Had they used the same bases in the form of salts, or in a neutral buffer solution they would have found the same bactericidal properties. This was actually done by Tilley and

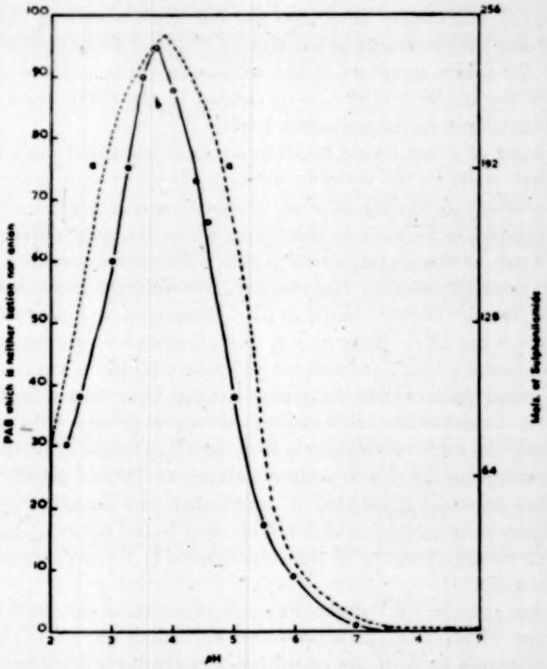


Fig. 3. Effect of *p*-aminobenzoic acid (PAB) in neutralizing the toxic action of sulphanilamide on *Polytomella caeca* at various pH values. (a) percentage of the PAB present as neither kation nor anion. (b) number of molecules of sulphanilamide antagonized by one molecule of PAB.

Schaffer in 1928 (60), but even then the obvious deduction was not made. However, in 1924 Stearn and Stearn (58) made their suggestion that organic kations (in particular those of the triphenyl-methane dyes) owe their antibacterial activity to a reaction with some anionic groups of bacteria to give feebly dissociated complexes of the type already discussed in Section 4 (iv) (see also 7 (iv)). Although the Stearns did not know the pK_a values of their dyes, they predicted that the salts of many strong (and fairly strong) bases would be found to be antibacterial because these would provide a sufficient supply of kations in the physiological pH range. They also showed that increasing the pH of the medium increases the antibacterial activity by bringing about increased (anionic) ionization of the acidic receptors of the bacterium. They pointed out that this alkalization of the medium must not be carried to the point where it begins to suppress the ionization of the antiseptic itself.

Rigorous proof of a connexion between kationic ionization and antibacterial action was first made in the acridine series (3, 7) which still provides the best examples of positive correlation between ionization and biological activity. Table 7 gives 26 examples of isomers in this series where the only difference between strong and weak antibacterial action is the difference between high and low degrees of (kationic) ionization. For example, five different mono-aminoacridines exist; of these, two are highly ionized at pH 7 because of an ionic resonance effect (5) whereas the other three ionize to only a small extent. It is evident from Table 7 that the two isomers which are well-ionized have a powerful antibacterial action whereas the three isomers that are poorly-ionized have only a feeble action. If we proceed to compare the well-ionized diaminoacridines with their poorly-ionized isomers, the same correlation is seen, and it is found again in the methylaminoacridines and in the chloro-aminoacridines, as Table 7 plainly shows. This correlation has been demonstrated in more than one hundred acridines and, provided ionization is kationic and is not allowed to fall below about 50 per cent at pH 7.3, the chemical nature of the substituents in the acridine nucleus make little difference (7).

Still more has been learned about the mode of action of acridines by the use of a test organism which withstands both acid and alkali (*E. coli*). This enabled a type of investigation to be made which has already been described for salicylic acid (Section 5 (i), above). This approach is complementary to that reported in Table 7. In such experiments, the ionization of the more poorly ionized acridines (*e.g.*, 3-aminoacridine) could be varied over the pH range of 5.5 to 8.3, and the effect on the organism observed. As a control, it was necessary to examine acridines which were fully ionized throughout this pH range (*e.g.*, 5-aminoacridine). The results of these experiments confirmed that the antibacterial action depended upon the kations rather than on the molecules (7). Further, the slope of the lines obtained when the logarithm of the antibacterial concentration of *kations* was plotted against pH (Fig. 4) revealed a direct competition between acridine kations and hydrogen ions. Naturally, no such correlation is seen when the logarithm of the antibacterial concentration of total drug (*i.e.*, kations + molecules) was plotted against pH (Fig. 5).

This correlation between kationic ionization and antibacterial activity exists not only in the acridine series but also in at least seven other hetero-aromatic series. These seven series comprise the various angular and linear benzacridines, benzoquinolines and phenanthridines. Further, provided that appropriate substitution is used to keep the flat area of the molecule above 38 sq. A (in order to assist adsorption by van der Waals bonds), this correlation is valid also in the pyridine and quinoline series (6).

TABLE 7

Dependence of bacteriostasis on ionization in acridine derivatives

Minimum bacteriostatic concentration for *Streptococcus pyogenes* after 48 hours' incubation at 37°. Medium: 10 per cent serum broth. pH:7.3.

-ACRIDINE	CONCENTRATION	IONIZATION PER CENT
5-Amino-	1 in 160,000	100
2-Amino-	80,000	73
3-Amino-	10,000	2
4-Amino-	10,000	2
1-Amino-	5,000	<1
2:5-Diamino-	1 in 160,000	100
2:8-Diamino-	160,000	99
1:5-Diamino-	80,000	98
2:7-Diamino-	160,000	76
3:7-Diamino-	20,000	3
1:9-Diamino-	<5,000	<1
5-Amino-1-methyl-	1 in 320,000	100
5-Amino-4-methyl-	320,000	100
5-Amino-2-methyl-	160,000	100
5-Amino-3-methyl-	160,000	100
3-Amino-5-methyl-	20,000	3
4-Amino-1-methyl-	20,000	1
1-Amino-9-methyl-	<5,000	<1
5-Amino-2-chloro-	1 in 160,000	96
5-Amino-3-chloro-	160,000	94
5-Amino-4-chloro-	160,000	86
5-Amino-1-chloro-	80,000	83
2-Amino-8-chloro-	40,000	33
2-Amino-7-chloro-	40,000	20
2-Amino-5-chloro-	<5,000	11
3-Amino-8-chloro-	<5,000	<1

An interesting conclusion to be drawn from Fig. 4 is that a smaller amount of any aminoacridine would be effective in wounds if they were prevented from becoming acid. Clinical studies along these lines were made by the Australian Army during the last war and, as a result, it was found that better results were obtained when sodium bicarbonate lavage preceded treatment with aminacrine (5-aminoacridine hydrochloride).

That a high degree of ionization may be necessary for the activity of certain antimalarials was first suggested in 1937 by Christophers (14, 15). It was shown by Gage in 1949 (26) that each of the four related substances ((III), (IV), (V) and (VI)) had two basic pK$_a$ values. The first constant of those printed below each formula (and measured at 25°) relates to the aliphatic side chain;

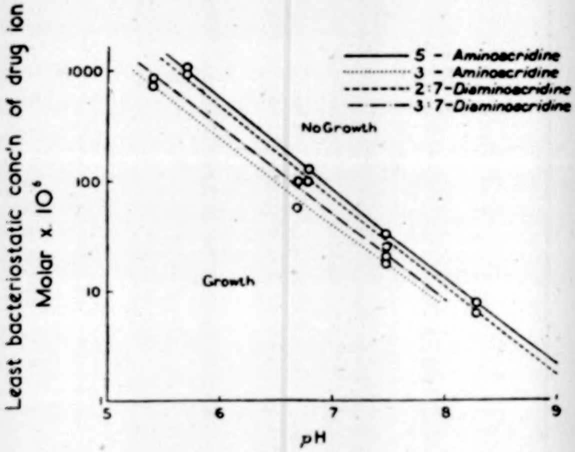


FIG. 4. Correlation in competition between hydrogen ions and acridine ions. Organism: *E. coli*.

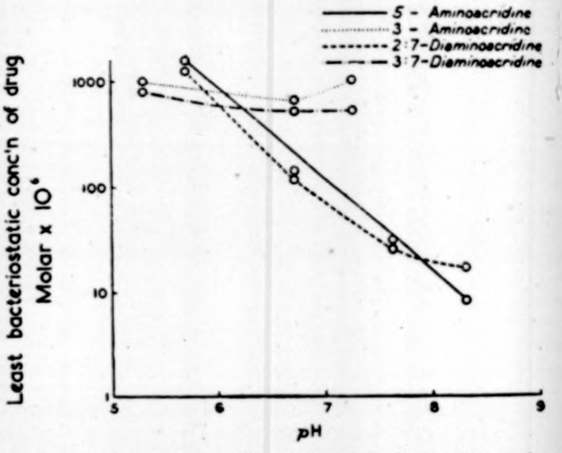
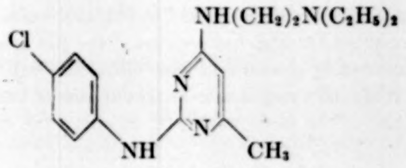


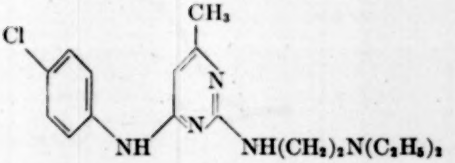
FIG. 5. Lack of correlation in competition between hydrogen ions and acridines (ions + molecules). Organism: *E. coli*.

it is evident that this is completely ionized, in the blood, as a kation at pH 7.3. The second figure refers to the pyrimidine nucleus and the amino-group attached to it which collectively form an amidine system (53). Substances (III) and (IV) are highly antimalarial, substances (V) and (VI) are almost without antimalarial action. Now at 37°, the second pK_a of substances (III) and (IV) becomes 6.4 (see Section 7 (i)) which, at pH 7.3, represents 11 per cent ionization as dikation. Likewise substances (V) and (VI) would give 1 and 0.04 per cent of dikations, respectively*. Rose based a general rule for predicting antimalarial activity in pyrimidines and diguanides (*e.g.*, proguanil) on these and similar results (53). However, he gave no clue as to whether analogues with higher second pK_a values were being prepared in order to obtain up to nine times as much of the desired ionic species.



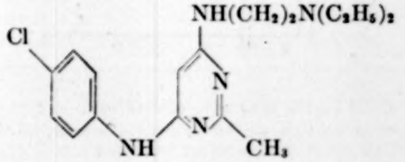
(pK_a: 9.05; 6.6)

(III)



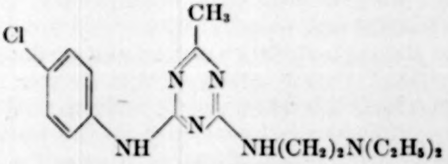
(9.6; 6.6)

(IV)



(9.3; 5.5)

(V)



(9.3; 4.05)

(VI)

* These calculations assume that the blood of the birds used in these experiments has the same pH as human blood. If it is not 7.3, but 7.0, the percentage of di-kation in substances (III) and IV) becomes 20, but those of the other substances are almost unaffected.

(iii) *Intermediate Cases*

A number of cases of biological action are known in which the molecule is far more active than the ion, but in which the ion does show some activity. Such behaviour is usually shown by phenols or other weak acids (4).

When submitting a weak acid to biological test, it is usually found that a constant amount of the substance is required to produce a standard response (regardless of the pH of the medium) at *all* pH values 1 unit or more below the pK_a. Under such conditions the ionization of the acid is slight (see Section 2 (vi)) and hence the biological effect is due to the molecule. This effect is illustrated on the left-hand side of Fig. 6. However if the pH is allowed to rise above the pK_a, an ever-increasing amount of the substance will be required to give the same response. When this response is analysed, one of two results is obtained, (a) a constant amount of the molecule is still required (*cf.* Section 5 (i) and Fig. 2) or (b) an ever decreasing amount of the molecule is required because the anion exhibits, to a limited extent, the biological action of the molecule. Result (b), which is illustrated in Fig. 6 by the action of benzoic acid on the mould *Mucor* (21), is far more common than result (a) (4).

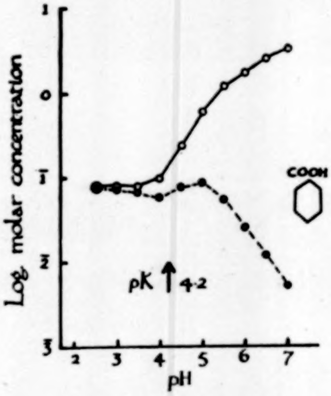


Fig. 6. The effect of pH on the concentrations of benzoic acid required to prevent the growth of *Mucor*. Upper curve: total substance (ions + molecules). Lower curve: molecules.

This method of plotting ionic data was developed by Simon (55) who found that the vast majority of those substances that are most active when least ionized nevertheless have ions which exert a small fraction of the activity of the molecules

Further examples of the increased efficacy of neutral molecules over their corresponding ions are provided in Table 8 which deals with the narcotizing action of various substances on the worm *Arenicola* (18). It can be seen that the effect of non-electrolytes such as chloroform is independent of pH. These non-ionizing substances provide valuable controls because they show that the changes of pH do not affect the worms. Passing on to the weak bases, such as cocaine, we observe that these become more effective as the pH is increased, *i.e.*, in proportion as their ionization is suppressed. Similarly we note that the weak acids (for isomeric barbiturates) are more effective as the pH is decreased; again this corresponds to the suppression of their ionization. Actually (although the authors did not do so), it is a simple matter to calculate that in this series the ions are making a small contribution to the toxic action. Similarly, it has been shown that the inhibitory action of nitrophenols on fungi is due principally to the neutral molecules but that the ions do make some contribution (55). The chemotherapeutic action of phenylarsenoxides upon spirochaetes has also been shown to be due principally to the neutral molecules (25).

TABLE 8

The connexion between ionization and the narcosis of Arenicola

(Minimal anaesthetic doses, in grams per 100 ml. of sea-water, rendering this worm immobile after 5 minutes.)

	pH 7.0	pH 8.0	pH 9.0
Non-electrolytes			
*iso*Propyl alcohol	2.5	2.5	2.5
*iso*Amyl alcohol	0.1	0.1	0.1
Chloroform	0.012	0.012	0.025
Chlorbutol	0.025	0.025	0.025
Weak bases (pK_a = *ca.* 8.5)			
Cocaine	0.01	0.005	0.0025
Procaine	0.002	0.001	0.0005
Butyn	0.001	0.0002	0.0002
Barbituric acids (pK_a = *ca.* 8.0)			
*iso*Amyl, ethyl- ("amytal")	0.006	0.025	0.05
Propylmethylcarbinyl, ethyl ("nembutal")	0.003	0.006	0.012
Diethylcarbinyl, ethyl-	0.006	0.012	0.05
n-Amyl, ethyl-	0.006	0.012	0.05

This type of activity is not confined to weak acids. An investigation was made of the anaesthetic action (on the rabbit's cornea) of five local anaesthetics (cocaine, procaine, stovaine, β-eucaine and benzylbenzoylecgonine), all of which are weak bases (61). An analysis of the results along the lines of Fig. 6 shows that the biological activity is almost proportional to the amount of molecule present but that the kation does make some contribution.

Conversely, not all acids act as molecules. It is not possible for strong acids, which can exist only as anions in the physiological pH range, to form molecules. There is good evidence that the antibacterial alkylsulphonic acids and sulphates (*e.g.*, sodium cetyl sulphate) act by forming ionic bonds with some of the essential kations of bacteria (27, 46, 51). These antibacterials are the oppositely-charged analogues of the kationic antibacterials.

Sometimes the buffer solution, in which an experiment is performed, has a slight toxic action. As buffers are traditionally (but not of necessity) the salts of weak acids, such toxic effects are commoner towards the lower pH values of each buffer's range. Sometimes this toxic effect is superimposed upon a curve which purports to show the effect of pH on the biological action of another substance (56). Such a source of error is revealed if buffers of various pK_a values are used. Thus the optimum for the growth of *Absidia orchidis* was found to be pH 4 when phosphate or citrate was used as a buffer; but with oxalate, which was slightly toxic, the optimum rose to pH 5 and with acetate, which was even more toxic, the optimum became pH 6 (22).

A curious situation arises among the sulphonamide drugs in that those members that are approximately 50 per cent ionized (as anions) at pH 7 are more powerfully antibacterial than such of their analogues as are ionized to a greater or lesser degree (8). One attempt at explanation assumed that the anionic form of the drug is the more potent but that the neutral molecule is essential to secure penetration (20). This explanation may well cover the phenols and other weak acids discussed above. However, in the case of the sulphonamides, the objection could well be made that these tests last for 24 hours or more, and hence good equilibration should be achieved; thus, much the same concentration of drug should be present both inside and outside the cell, almost regardless of the pK_a. Moreover, many closely-related substances which cannot dissociate into anions are valuable antibacterials, *e.g.*, sulphaguanidine and the various diphenylsulphones. A better explanation (8) has been put forward, *viz.*, that the really important thing is the size of the negative charge (on the SO_2-group) which appears to pass through a maximum value for those sulphonamides whose pK_a is approximately 7. This charge may assist adsorption through an ion-dipole bond on to the affected receptor, which appears to be the enzyme normally responsible for converting *p*-aminobenzoic acid to a member of the folic acid family.

6. THE IONIZATION OF RECEPTORS

A biological receptor, just as originally postulated by Ehrlich, is regarded as a molecule, or portion of a molecule, whose normal function in metabolism can be blocked by combination with an appropriate drug (*cf.* 4). There is evidence that receptors are often coenzymes or the protein components of enzyme systems.

The receptors which are relevant to the present review are those which can combine with drugs by ionic bonds, a type of receptor which only ionizable drugs can block. The pK_a values of various receptors cannot be predicted in advance of experiment for their chemical nature is largely unknown. Obviously, kationic drugs must combine with anionic receptors, but these may have pKs of 2 (due to the presence of phosphoric acid or amino-acid groups), 3 to 6 (carboxylic acid groups), 10 (tyrosine, purines, cysteine groups), etc. Kationic receptors could have pKs of 5 (pyridine groups), 7 (histidine), 10 (lysine), 13 (arginine), etc.

(i) *Receptors Outside Cells*

By no means are all receptors within cells. For example, amoebae are narcotized when undecane is injected into the external membrane, but not when it is injected into the amoeba (44a). The frog's heart is affected (in opposite ways) by acetylcholine and methylene blue without penetration of the cells taking place (16). These and many similar observations are in harmony with current knowledge that the outside of a cell is rich in enzymes concerned with assimilatory and other processes. For example, yeast is known to have adenosinetriphosphatase and several hydrolases on the outer surface (54, 59).

The pK_a of a receptor on the outside of cells can often be studied by measuring the response to drugs over a range of pH values, provided that (a) the cell is known to be unaffected by the pH changes and (b) that the ionization of the drug does not change within this range (see Section 2 (vi)). For example, the effect of 5-aminoacridine on *E. coli* between pH 5.5 and 8.3 (see Fig. 4) shows that the drug is combining with a receptor which is ionizing more and more as the pH is raised. The drug has a pK_a of 10 and hence is completely ionized throughout this pH range. The rate of this increase in ionization (of the receptor) with increase in pH is undiminished at pH 8.3. As the increase would lessen when the pK_a of the receptor was reached, this pK_a must be 9 or more (3). That the receptors for kationic antibacterials are on the outside of cells is known from two facts: (a) examples with lipophilic groups are no more effective than those without such groups and (b) examples which yield 70 per cent of kation at pH 7 are no more active than those which yield practically 100 per cent (*cf.* Section 4 (iii)) (3).

Work on determining the pK_a of external receptors in this way is still in its infancy but has great potentialities for development from its early roots in the work of Stearn and Stearn in 1924 (*cf.* Section 5 (ii)).

Not all workers have given due consideration to the possibility that the receptor may change its degree of ionization as the pH is changed. For example eucupine (a derivative of quinine) was found to be more lethal to *Staph. aureus* at pH 8.6 than at 6.1 (45). After showing that the change in pH was not in itself injurious to the organism, the authors concluded that the active form of the drug must be the molecule and that it should be possible to make improved drugs in this series by the introduction of base-weakening groups into the molecule. They did not refer to the possibility that the effect of alkalinity on the action may be to increase the ionization of the relevant receptor; if this is so, the introduction of base-weakening groups would be undesirable. Again, in 1939 the relative toxicity of tyramine, benzylamine (and a number of related amines) for staphylococci was contrasted with that of their hydrochlorides in unbuffered media (see Section 2 (i)). Without measuring any pK_a values, the author concluded that the antibacterial action was due to the molecules rather than the kations. Clearly, the design of these experiments does not permit any such conclusion to be drawn.

Again, the respiration of avian red blood cells (whole or haemolysed) has been found to be inhibited 2.5 times as strongly by quinine at pH 10 as at pH 5. Because quinine has a pK_a of 8.4, it was concluded that the inhibition was caused by the molecule and not by the ion (by the base quinine and not by quinine salts, as the authors put it). The possibility that an acidic receptor ionizes at the higher pH (whereupon it can bind more quinine kations) is not mentioned (52).

(ii) *Receptors Inside Cells*

When any of the above tests suggests that a receptor is *within* the cell, ionization studies become more difficult. Obviously the pH surrounding the receptor is of prime importance, yet little is known of the pH within animal cells. Many experiments have been performed with indicators to determine the pH of protoplasm, but the results obtained are only a record of the average pH values of protein strands and other internal surfaces (see Section 7 (vi)). Some of these surfaces are likely to be poorer or richer in hydrogen ions than others, and they may not be at the pH of the internal bulk phase. This indicator method has suggested, rightly or wrongly, that the internal pH of animal cells tends to lie between 6.5 and 7. As the exact location of internal receptors is not known, this rough estimate may serve until more detailed knowledge is available.

That the pH within cells may roughly correspond to that of buffers in which they are placed is suggested by the following experiment. The tyrosine-decarboxylase of *Strept. faecalis* has itx maximal activity *in vitro* at pH 5.5. In the intact bacterium, it is only moderately active when the external environment is neutral or alkaline, but it becomes most active when placed in buffer at pH 5.0 to 5.5 (26a).

Thus it is desirable in pharmacology to work with a buffer, even when the receptor is known to be an internal one. Moreover the external effect of a buffer is desirable because it brings about uniform conditions by (a) presenting the drug to the cell in a standard state of ionization, regardless of the form in which it was supplied and (b) maintaining in a standard state the mechanisms for the adsorption of the drug to the cell surface and its penetration into the cell.

The success of buffering depends on the proportion of cells with which the buffers come in contact. In bulky tissue-preparations, this proportion may be very small, and the drugs can reach cells remote from the surface only after passage through other cells or intercellular fluids. The results from such experiments are not comparable with those obtained from experiments based on thin slices of tissue in which all the cells are in direct contact with the drug at a known pH (56).

7. FURTHER ASPECTS OF THE CHEMISTRY OF IONIZATION

The brief account of the chemistry of ionization given in Section 2 was intended to serve as a background for sections 3 to 6. However, there are other aspects of ionization chemistry which deserve mention because of the influence which they may exert upon the design of pharmacological experiments.

(i) *Temperature Effects*

The neutral point and many ionization constants vary with temperature. The neutral point is half the pK_w (defined in Section 2 (iv)). Thus neutrality occurs at pH 7.0 at 25°, but at pH 6.8 at 37°.

Bases become weaker as the temperature rises, and this effect is most marked with strong bases. Thus 0.011 is to be substracted for a base of pK_a 3 for each degree increase in temperature; for a base of pK_a 11 it becomes 0.022. Carboxylic acids hardly vary in pK_a with temperature changes, but some other acids show changes similar to those of bases.

(ii) *Zwitterions*

So far, no mention has been made of zwitterions, *i.e.*, ionic forms which carry both positive and negative charges. In discussing whether a substance is zwitterionic or not, the pH range in which the information is required must be specified, for a sufficiently alkaline solution will change the zwitterion to an anion, and a sufficiently acid solution will change it to a kation.

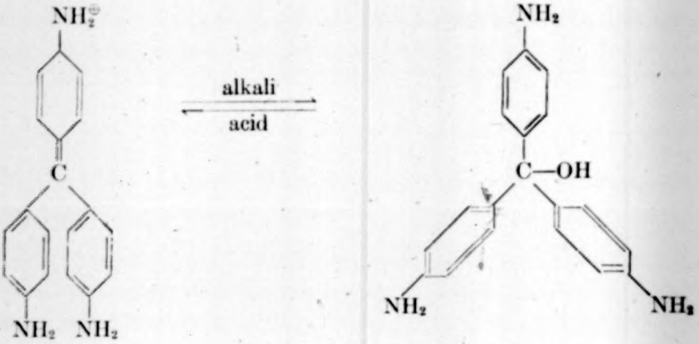
For example, the acidic group in glycine has a pK_a of 2.2, and the basic group has a pK_a of 9.9. It is evident from Table 4 that at pH 1.2, only 10 per cent of the acidic groups (but *all* of the basic groups) will be ionized; hence at this pH, 10 per cent of the substance will be present as zwitterion and 90 per cent as kation. These calculations can be repeated at any other pH values which are of interest and it will be found that glycine is almost entirely zwitterionic between pH 3.3 and 8.9. Thus, a substance is 90 per cent or more in the zwitterionic state when the pH is at least 1 unit above the acidic pK_a and at least 1 unit below the basic pK_a. If this rule is applied to *p*-aminobenzoic acid (acidic pK_a 4.8; basic pK_a 2.7), it will at once be seen that this substance, unlike glycine, is exclusively anionic in neutral and alkaline solutions. At pH 3.8, solutions of *p*-aminobenzoic acid contain 90 per cent (neutral) molecules, 9 per cent zwitterions, and 1 per cent or less of kations or anions.

Zwitterions are usually rather inert pharmacologically. For example, histidine has none of the marked physiological properties shown by the closely related kation, histamine. The pharmacological properties of the histidine kation are unknown as this ionic species exists in quantity only below pH 2.

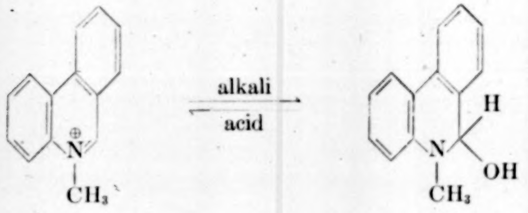
Again, the vinyl group of quinine can be oxidized to a carboxylic acid, thereby converting a kation to a zwitterion. Concomitantly, the antimalarial properties disappear. When this acid (quitenine) is esterified, the substance of necessity becomes kationic again; this ester is strongly antimalarial (30). Similar examples have been found in the acridine series (3).

(iii) *Pseudo-Bases*

The time taken for ionic equilibria to occur, in solution, is so exceedingly small that ionic reactions may be regarded as instantaneous. However, a limited variety of kations are capable of combining *covalently* with hydroxyl ions to give non-ionized substances known as "pseudo-bases" from which the original kation can be regenerated by acid. Although the reaction is governed by mass-action relationships, anything from an hour to a week may be needed for equilibrium to be achieved. The most common examples of pseudo-bases are furnished by heterocyclic quaternary compounds and by triphenylmethane dyes. Suitable methods for calculating the equilibrium ionization constants (expressed as pK_a^E) and the velocity constants at which equilibrium is obtained were recently devised and have been applied to the triphenylmethane dyes (29). These dyes undergo the transformation exemplified by parafuchsin, of which the kation (VII) is vulnerable to attack by hydroxyl ions, which gives the tertiary alcohol (VIII). Likewise heterocyclic quaternary compounds undergo the transformation exemplified by the kation of phenanthridine methochloride (IX), which gives the secondary alcohol (X). Several trypanocides belong to this class of substances.



(VII) (VIII)



(IX) (X)

It is not possible for the pharmacologist to correlate ionization with biological response if pseudo-bases enter the picture, unless he has first ascertained the pK_a at equilibrium and the time taken to achieve equilibrium. It may be advantageous to achieve equilibrium at a carefully chosen pH value before bringing the drug solution and the biological test-object together. It may be well to bear in mind that many secondary alcohols of the type (X) are easily oxidized to the corresponding ketones; each individual case can be tested for absorption of oxygen in a Warburg manometer. Few pK_a^E values of heterocyclic quaternary compounds have been obtained (29, 44) but it is evident that these are often much weaker bases than aliphatic and aromatic quaternary compounds; some aminotriphenylmethanes are very weak bases indeed. Unfortunately attempts have been made to explain the action of trypanocidal quaternary compounds without taking these factors into account (*e.g.*, 49).

A considerable amount of work has been done with triphenylmethane dyes in the field of therapeutic interference, and the experiments have involved estimating the amounts of various dyes taken up by cells or displacing one another from cell surfaces. Such colorimetric analyses have been carried out without any of the precautions outlined above and are of questionable validity. Doubts arise even more strongly when alcohol has been used for extracting the dyes (36) as it rapidly etherifies alcohols of the types (VIII) and (X), thus furnishing a further barrier to equilibrium.

(iv) *Thermodynamic Activity Effects*

As was explained in Section 2 (i), anions and kations tend to form some ion-pairs in concentrated solutions and thus behave as though a (usually minute) fraction of their total concentration were absent. This thermodynamic activity effect (the activity being slightly less than the concentration) is reflected in pK_a values, both acids and bases appearing to be weaker in strong solutions. For example, acetic acid has the following pK_a values: 5.10 (M); 4.86 (0.1M); 4.79 (0.01M); 4.76 (0.001M). The value at infinite dilution can be calculated to be 4.75, and this is known as the thermodynamic pK.

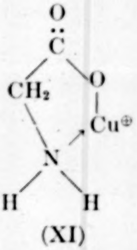
Ions of formula-weight over 200 have a greater tendency to form ion-pairs (or even more complex aggregates) than the simple ions which we have been considering; hence these heavy ions show considerably greater activity effects, *i.e.*, the proportion of free to bound ions becomes very low. This must often be the case when a biologically active ion has combined with an oppositely charged receptor, particularly if the latter is part of a protein or a nucleic acid. The better the fit between the non-ionized parts of the two ions, the higher the activity effect (6). Although such combinations are as completely ionized as sodium chloride (Section 2 (i)), they provide only small amounts of free ions, the equilibrium being governed by a mass-action equation (see Section 4 (iv)). The size of K_s, the constant governing such an equation, is a measure of the strength of the non-ionic bonds cooperating with the ionic bonds in such a union. Such non-ionic bonds may be either hydrogen bonds, van der Waals' forces, or both, but they are not likely to be covalent bonds.

Drugs which are desired to provide a depot effect, *i.e.*, are required slowly to liberate a therapeutically-active ion, can be formed by combing such an ion with an oppositely charged ion, both being of fairly high molecular weight. Such compounds are slow to release the curative ions, because of their large thermodynamic activity effect. In addition, they are often (but not necessarily) sparingly soluble. Procaine penicillin is a well-known example.

(v) *The Binding of Metallic Ions*

Organic molecules having two neighbouring groups (one being an anion and one an electron donor such as a nitrogen or oxygen atom) form salts (with metallic ions) which show very high thermodynamic activity effects. That is to say, they are in equilibrium with very small amounts of the free metal ions. For example, the antiseptic 8-hydroxyquinoline combines with copper ions so that many millions of cupric kations are bound for each one that is free.

The name "chelation" was coined by Morgan and Drew (48) to describe this phenomenon, the name being derived from *chela* the crab's claw, in allusion to the shape and function of the metal-seizing pair of groups. A typical example of chelation is the union of one cupric ion with two anions of glycine to give the tightly-bound complex (XI). Complexes of this type play an enormous part in biochemical processes. Their equilibria with the free ions from which they are derived can be described by mass action equations and simplified methods for calculating the required constants have recently been published (2).



(XI)

(vi) *The Zeta Potential* (ζ)

Those who are familiar with hydrogen-ion measuring equipment know that the $H^{\oplus}$ concentration is measured as a potential difference between two electrodes immersed in the solution. The greater the hydrogen ion concentration, the greater this potential. Conversely, if a potential exists between water and a surface immersed in the water, then the hydrogen ion concentration on the surface must be different from that in the water. Most biologically important surfaces contain polar atoms (O, N or S), and hence a small potential exists between the surfaces and water. This is the electrokinetic, or zeta, potential (ζ). Hartley and Roe (35) derived the following equation:

$$\text{pH (surface)} = \text{pH (solution)} + \frac{\zeta}{0.06} \qquad \text{(vii)}$$

(where the zeta potential is measured in volts). Zeta potentials of as much as 0.1 volt are common. Using a salt of cetanesulphonic acid to provide the necessary surface (in the form of colloidal particles dispersed in water), the authors showed that, while the pH of the solution was 8.0, that of the surface was 6.3. The difference between the two pH values decreased with rising concentration, the temperature being maintained at 30°. As biological surfaces are usually anionic, it must be expected that they will be at a lower pH than that of the buffers bathing them. Similarly, kationic surfaces should be at a higher pH than the buffers. What the magnitude of these differences is for proteins, nucleic acids and other biologically interesting colloidal material is almost unknown, but is not likely to exceed the values obtained in the above experiment.

The great majority of known dyes tend to congregate at surfaces, hence the limitations of indicators when used to determine the pH inside cells (*cf.* Section 6 (ii)).

(vii) *The Determination of* pK_a *Values*

From time to time the pharmacologist will need to know pK_a values which have not yet appeared in the literature. These values may be determined conductimetrically, spectrographically or potentiometrically. The last method is least laborious. About 0.1 gram of substance is required; it must be completely pure and can be recovered unchanged after the titration (which is best carried out by a chemist who can apply all the necessary safeguards and corrections). For the potentiometric method to be applicable, the substance must be sufficiently soluble, both salt and molecule. It is difficult to find the pK_a of a sparingly soluble substance by titration if the value is low (it is usually not practicable to find a pK that is lower than the logarithm of the dilution employed). By the spectroscopic method, much more dilute solutions can be dealt with, but this method can be applied only to a substance whose ion and molecule have distinctly different spectra.

The following is a typical equation used in potentiometry (it is actually that for a weak base):

$$pK = pH - \log \frac{[B] + [H^+]}{[BH^+] - [H^+]} \tag{viii}$$

where [B] and $[BH^+]$ are the apparent values calculated from the amount of acid added.

The pharmacologist who sends a specimen away for pK_a determination should ask to have the values reported to him at nine equidistant intervals (*viz.*, at 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent neutralization). Excluding the two extreme values (10 and 90 per cent), the other values should agree within ±0.06. If this is not so, impurities are probably present, but occasionally the substance has decomposed during titration as is revealed by different values being obtainable upon back titration. Small credence should be given to pK_a values determined at "50 per cent neutralization" only, a method open to undetectable errors.

When two pK_a values, in the one substance, are separated by 3 units or less, the pH at 100 per cent neutralization of the first group should be numerically the average of the two pK_a values. When the pK_a values are 1.5 or less apart, equations of the type (vii) will not suffice, and the method of successive approximations must be applied. (For the method of successive approximations, see reference 9, p. 197.)

8. CONCLUDING REMARKS

Consideration of the principles discussed in this review makes it clear that it is highly advantageous for biologists to know the ionization constants of substances which they are investigating.

No less important is the next step which is to discover which is the most effective, the ion or the molecule. This may be done in two ways, which are not alternative but complementary. In the first approach, the pK_a of the drug should be varied by appropriate substitution, taking full advantage of the chemist's new-found ability to produce substances of any desired pK_a in almost any series. The advantage of this approach is that the ionization of the receptor is not interfered with, and the living cells are not removed from their optimal pH. The disadvantage is that the chemical change in the molecule may, of itself, be responsible for a changed biological response; this difficulty can be avoided by working with *two* analogues at each desired pK_a, and accepting the results only if they agree. In the second approach, the composition of the drug should be kept constant, but the pH of the medium varied. This has the advantage that the living cells are exposed to only one drug, but the effect of pH changes on the receptor and on the viability of the cell itself must be independently examined.

Studies of this kind can add greatly to the scientific knowledge derivable from a biological experiment (and can assist in designing more meaningful experiments) because one of the commonest of variables is brought under control.

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NEUROPHARMACOLOGY OF PERIPHERAL NERVE*

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I. INTRODUCTION

Active research in peripheral nerve physiology is now over a century old. Like the centenarian, it tends to become set in its ways. The vast body of accumulated experience is often hard to translate for the benefit of those in other fields, who grow impatient with the language, the compulsions and the scholastic disagreements of the axonologists. But present needs, on the part of the neurophysiologist who requires chemical tools for his work as well as on the part of the pharmacologist who wishes to find mechanisms of action, demand that someone build a neuropharmacological bridge. The present reviewer, poorly equipped for this pontification, will try to assemble the most relevant data and opinion from the works of those neurophysiologists who have contributed most heavily to the pharmacological field in the past two decades—in particular, Lorente de Nó and his colleagues (98–100, 165, 166, 182–189), Gerard's group (32, 33, 45, 73, 74, 103–107, 175, 176, 210, 236, 265, 266), Nachmansohn and associates (41, 42, 120–122, 159, 205–207, 234), Bronk's laboratory (34–37, 52, 167, 168), Prosser (221–223a), Rosenblueth *et al.* (177, 229–234), Welsh and others (114, 239, 240, 287, 288), Höber (135–139), Crescitelli (56–60) and Shanes (245–247). It will be impossible to cite everyone who has worked on drug action on nerve, and some contributions, although important, will be passed by to permit space for discussion of certain key problems in neurophysiology.

It is the purpose of this review first to survey those properties of peripheral nerve which may be studied to the advantage of pharmacology, and then to enumerate the actions of specific drugs of interest—particularly those which have actions of relevance to normal neuronal function. Unfortunately, except for local anesthetics, little of this information has practical significance as yet for the clinical applications of pharmacotherapy. It will have to justify itself at present for whatever theoretical overtones it can offer.

II. PROPERTIES OF NEURONES

This section is not planned as a consistent summary of theoretical neurophysiology. Instead it is intended to outline those properties which can easily be measured and which are potentially modifiable by drug action. For theoretical summations on the relationships of properties of nerve fibers, a number of reviews and monographs, old and new, should be mentioned (12, 20, 23, 24, 26–28, 36, 67, 75, 82, 101, 105, 106, 120, 133, 135, 137, 146, 152, 164, 179, 184, 190, 202, 206, 214, 218, 223, 223a, 225, 238, 251, 269, 291). The following subdivision of properties into "resting" and otherwise is made with some hesitation, because modern investigations show beyond a doubt that the non-conducting fiber is considerably more than an osmotic sausage-casing. Indeed it can be said that those same processes which enter into propagation and recovery are most probably operative but in balance in the "resting" state. However, it is somewhat convenient to differentiate between steady states on the one hand and explosions on the other.

A. "*Resting*" *Properties*

1. *Structural features.* Only sporadic attention has been paid to visible alterations of axone structure as a function of experimental procedures, but recently there has been an increasing interest in this field. Following Flaig's report (95) of opacity changes associated with stimulation of large invertebrate fibers, Tobias (253, 265, 266) and Hill (134) have studied light-scattering and volume changes, and Tobias has related these to effects of cations. Lorente de Nó (184) has made occasional observations on frog axones, noting, for example, the swelling and disintegration of the myelin sheath with high calcium concentration, and the tendency of myelin to flow and surround the nodal incisures in old preparations. Spectophotometric studies during activity have been made by Minz (200) and von Muralt (280) for the detection of liberated substances. The tissue culture methods of Pomerat (219, 220, *cf.* also 15), which have now been applied to adult neurones and glia of brain and cord, offer sensitive preparations for visual study of drug action on regenerating cells. It would be worth while to repeat some of the gross observations by Marinesco (see 13) on such preparations. In view of the increasing interest in neuro-regenerative substances, and the demonstration by Weiss (286) that there is a perpetual normal flow of axoplasm from the cell body, it would be important to gather information on the pharmacology of isolated intact neurones in culture.

The variations in size and type of axones offer a perplexing choice of material for studies in pharmacology, but certain fundamental qualities are universally present. Although the fiber diameters may vary from a micron in vertebrate unmyelinated fibers to a millimeter in invertebrate giant fibers (48, 223, 288), all have in common a complex lipoprotein surface, whether vanishingly thin or measurably thick, and densely or loosely packed (48, 67, 241). Both the lipoprotein surface and the gelatinous axoplasm show structural orientation (67, 69, 241). Since all modern theories of excitation demand active physical and/or chemical changes in the membrane, and some require participation of the core as well (184), there is probably much of value to be learned from visual observation during drug application. Certain differential features, such as the nodes of vertebrate myelinated axones or the septa of certain invertebrate giant fibers, appear to be of less functional interest. At any rate, modern microscopic techniques, such as polarization or phase microscopy, spectrophotometric and scatter measurements, and the use of time lapse cinematography, are worth further exploration by pharmacologists.

2. *Permeability.* It is of prime importance to determine whether substances under study can penetrate into the nerve fiber and, if so, whether they in turn alter the permeability of the fiber for other substances. Many controversies have hinged on the question of penetration of an ineffective drug, for example, acetylcholine (33, 106, 184, 206), and permeability changes are often invoked to explain drug actions (*e.g.*, 97). To perplex the situation further, drugs are sometimes assumed to act by adsorption upon a surface membrane (23, 114), in which case evidence concerning penetration into the axoplasm would be irrelevant. There are available a number of excellent reviews dealing with permeability (39, 67, 135, 137, 160, 214, 238, 275).

Actually it is difficult to define the site of the supposed surface where critical functions are thought to occur in neurones. Lorente de Nó (184) has given reasons for considering that there are three such sites, that the axoplasm itself is involved in depth, and that the myelin-extracellular interface is not critical. For the present, therefore, it might be better to think of a cortex rather than an interface, and to assume that agents which act upon nerve must penetrate some distance toward the interior.

Earlier studies seemed to indicate that neurones were impermeable to anions and to sodium (19), and passive penetration of ions has been assumed even recently to determine the distribution of electrolytes, with potassium inside and sodium outside (53). However, investigations with radioactive ions have made it clear that there is a metabolic pumping action in operation to separate ions. Krogh (160) and Ussing (275) among others have given evidence for the existence of active transport of substances in cells, particularly in the case of sodium. Since the transfer rate of radioactive sodium appears to be even faster in nerve than that for potassium (284, 292), it is apparent that an active mechanism, dependent upon metabolic processes, is necessary for the maintenance of the observed electrolyte distribution. The high temperature coefficient for penetration of phosphate (93) would also seem to indicate active transport. In brief, even where a particular substance seems to penetrate very slowly if at all, as judged by gross chemical methods, there exists the possibility that it does indeed penetrate but is pumped out at a rate which keeps the intracellular concentration low or constant.

For any drug group there appear to be a number of factors determining penetration rate into nerve, as into other tissues. In general, nonelectrolytes are taken up more easily than electrolytes, smaller more easily than larger ions or molecules, undissociated more easily than dissociated molecules, tertiary more easily than quaternary ammonium compounds (137, 223a). Nonpolar compounds which are more lipid-soluble tend to penetrate more readily than polar, but there are special considerations concerning the long-chain fatty acids and other polar-nonpolar compounds which are thought by some to play a special role in nerve (135–138). It is chastening to note that in some cases even proteins, such as botulinus toxin (6, 126), with a molecular weight of a million and bristling with polar groups and free valences, nevertheless manage to penetrate nerve fibers and exert characteristic actions on enzymes that must lie deep below the surface.

In view of the ease with which many substances can now be prepared with radioactive or heavy isotopic tracers, and the encouraging results which have been obtained by tracer studies in nerve (68, 93, 117, 121, 155, 156, 204, 234, 284, 292), it seems that much of the older information on permeability, based on gross chemical analysis or even on indirect estimation by observation of ultimate effects of drugs, will be in need of revision.

3. *Resistance of membrane and core.* Membrane resistance is reciprocally related to permeability for ions, and thus has significance for practical determination of drug effects, as well as for a study of the basic electrical properties of axones. Two general methods have been evolved for measuring membrane resistance. The method of Curtis and Cole requires the actual penetration of the axone by a microelectrode and the use of alternating current as the signal in a bridge circuit, for which the membrane serves as one arm (49–51, 63, 285). The other utilizes external electrodes and the passage of a steady subthreshold current through the fiber or nerve; the necessary data are derived from measurements of the rate of fall of the steady electrotonic potential in the extrapolar region and the current flow in the interpolar region (140–144, 153, 184, 284). The basic theoretical discussions necessary for an understanding of the latter method will be found in the works of Hodgkin and Rushton (144) and Lorente de Nó (184).

The estimated membrane resistance varies widely among axones, from 15,000 ohm cm^2 in myelinated peroneal fibers of the bullfrog (184) to 300 ohm cm^2 for giant fibers of the squid (51). Strangely enough, if one multiplies the membrane resistance by the axone diameter for several types of fiber, one obtains an approximately constant value, the resistance dropping regularly with increasing diameter over a fifty-fold range. The relationship is such that the flow of ions across the membrane tends to remain constant per unit volume of axone regardless of size. Since nerve metabolism per unit volume does not vary widely among various fibers, and membrane voltages are similar despite size, the significance of the diameter-resistance relationship becomes obvious. However, such a relationship tends to set a limit to fiber size, for above a millimeter the membrane would have to approach axoplasm in permeability, and below a micron there would be little room for axoplasm, in order to satisfy the above conditions.

Axoplasm itself has a relatively low resistance, although several times greater than that of the usual extracellular fluid.

It is disappointing to note that this methodology and theory have received practically no pharmacological application except insofar as potassium has been found to decrease membrane resistance (141, 142, 156). For ordinary purposes a relative estimate, based on the measurement of extrapolar electrotonic potential at a few points, should suffice to indicate the general direction of change with drug action. In view of the importance attributed to the axoplasmic core (184) and the previously discussed physical changes during activity, estimates of drug-induced changes in axoplasmic resistance might also be useful.

4. *Capacity and inductance.* All electrical models of nerve demand, as one of the circuit elements, an equivalent capacity—that is, the property of storing a quantity of electrical charge. The membrane capacity can be estimated either by phase analysis, using the frequency method of Cole and Curtis (50), or by the method of Hodgkin and Rushton (144) and Lorente de Nó (184), based on the time rate of rise or fall of the electrotonic potential in conjunction with the method for determination of membrane resistance. Myelinated vertebrate fibers have an equivalent capacity of approximately 0.01 microfarads/cm^2 (184), while most other fibers are reported to be of the order of 1.0 microfarad/cm^2 (49–51, 140, 144, 284). The difference is presumably related to the depth of the myelin layers in vertebrate fibers, giving a series arrangement of equivalent condensers.

There is less agreement on the presence or significance of an equivalent inductance in nerve models. An inductive reactance may be assumed in a circuit element when a potential appears across it which is proportional to the rate of change of current. Such an effect appears in the overshoot of the action potential of squid fibers, and in various rhythmic phenomena. Cole (49) has estimated the apparent inductance of squid fibers, but Lorente de Nó denies the validity of such estimates on theoretical grounds (184). Practically, an inductive reactance assumes importance only when the membrane resistance is low and damping therefore minimized.

Since estimates of both capacity and inductance are dependent upon measurements of resistance, it is not surprising that drug effects have been neglected.

5. *Characteristic length, time and frequency.* The passive electrical properties already described may be manipulated to evaluate certain quantitative features of nerve which are of importance to excitation theory. The natural unit of length, λ, is the distance over which a steady electrotonic potential falls to $1/e$ in the extrapolar region. It is simply related to the internal, external and membrane resistances by the relation $\lambda^2 = r_m/(r_e + r_i)$. For frog nerve its value is given by Lorente de Nó as 3 mm, and others have reported similar values (152, 184, 238). For invertebrate preparations the values are lower since the membrane resistance is lower (144, 284), and one must assume that the same would hold true for the unmyelinated soma and processes of vertebrate central neurones, where a high value would lead to serious problems in neuronal interactions.

The natural unit of time, τ, is estimated from the time required for the electrotonic potential to reach 85% of its final value at a polarizing electrode. For frog nerve it is reported by Lorente de Nó as 0.2 msec (184). It corresponds to the product of the membrane capacity and resistance, $c_m r_m$. Since invertebrate fibers have in general a larger capacity, and a resistance reciprocally related to size, one finds values for giant fibers approaching in brevity those of vertebrate nerve.

A combination of inductance and capacity imparts a characteristic frequency to a circuit. Brink *et al.* (34) have estimated a basic local oscillatory period of 3 msec in calcium-free squid axones, which agrees well with the characteristic undamped frequency derived by Cole (49) from inductance and capacity measurements. Brink found a similar periodicity of 6 msec for frog myelinated fibers. It would be of interest to determine drug effects not only on characteristic frequency but also on damping, since the latter is related to membrane resistance.

6. *Electrotonic potentials and polarizability.* Lorente de Nó (184) has used the measurement of electrotonic potentials extensively in studies of drug action. The method is simple in that one records the voltage developed between two widely separated electrodes while applying rectangular pulses of known current through another pair. The resultant record of voltage exhibits three distinct components, corresponding presumably to three polarizable layers within the fiber, and related to three differentiable components of the membrane resting voltage. Although such measurements are among the oldest in neurophysiology, Lorente de Nó has developed them to a high state of perfection. The method was primarily focussed on the solution of basic neurophysiological problems, but the variety of drugs used as tools attests to the sensitivity of this method for pharmacological study. The slowest electrotonic component is particularly vulnerable to chemically-induced changes. Since changes may be observed under conditions of conduction block or depolarization, the range is less restricted than for methods involving propagation of impulses.

7. *Membrane voltage.* Over a century ago Matteuci (197) discovered that voltage differences could be measured along the length of dissected muscle, and DuBois-Reymond (75) showed that an injured portion of nerve was electronegative to the intact surface. Helmholtz (129) and later Hermann (131) dealt with the various alternative possibilities of origin of injury potentials. Hermann believed that they were produced at the time of injury, while Bernstein (19) contended that the pre-existing difference of potential across the nerve membrane was conveniently revealed by the injury. Bernstein and later Cremer (55) postulated that the resting membrane potential was attributable to the difference in concentrations of potassium inside and outside the nerve, which was believed to be impermeable to sodium and anions. This passive explanation still finds considerable acceptance, although the evidence against such a simple mechanism has considerably expanded in the last two decades. Koch (157) and Gerard (103) were the first to demonstrate the dependence of the membrane potential upon oxidative metabolism, and it is now generally accepted that, regardless of the precise mechanism of voltage production, it can be maintained by the nerve only through the performance of metabolic work.

The method of measurement of membrane voltage by direct insertion of a microelectrode was first developed for use in large plant cells (28, 215), and the first such measurements in nerve were made by Cole and Curtis (50) in the giant axone of the squid, over half a millimeter in diameter. For investigations of smaller frog muscle fibers, Ling and Gerard (175, 176) developed an extremely fine KCl-filled glass capillary, less than a micron in tip diameter. The method was applied to cardiac muscle by L. A. Woodbury and to frog sciatic A fibers by J. W. Woodbury (293). Weidmann (284) has applied this more refined method to squid axones as well as to mammalian heart, and many others are now utilizing microelectrode recording for other tissues. Judging from the stability of membrane voltages recorded by various authors, the technique is relatively noninjurious and gives a prompt and accurate evaluation of membrane potential.

The voltages recorded by puncture methods are in general greater than those previously reported by indirect methods, running usually in the range from 60 to 100 mV for all tissues which have been studied. The technique also has established beyond a doubt that the voltage is developed across something corresponding to a membrane, since the establishment of potential is sharp during the initial puncture and relatively constant on further manipulation, although minor variations with insertion suggest that the voltage-producing layers have a finite thickness.

One disadvantage of the micromethod for pharmacological purposes in procedures on vertebrate nerve is the difficulty in maintaining a given insertion in a particular fiber while altering fluid. For this reason many prefer to continue the use of older gross methods, involving a comparison of treated and untreated surfaces, or measuring against a reference electrode in an area depolarized by injury or by isotonic KCl. Lorente de Nó and his coworkers (98, 100, 182, 189) have made extensive observations of drug actions on frog nerve in this manner, as has Shanes (245–247) with invertebrate nerve. Although Shanes and a number of others (54, 89, 90, 125, 141) place primary emphasis on the role of potassium in maintaining membrane voltage, Lorente de Nó inclines toward the view that depolarizing agents including potassium act upon oxidative mechanisms which maintain voltage in some other manner. Hodgkin (141) assumes that the metabolic work is done in pumping out sodium ion. The experimental fact remains, as shown by Lorente de Nó (184), that in frog sciatic nerve the membrane potential is maintained for long periods of time in the absence of any inorganic ion species in the external fluid, and that the nerve requires only oxygen and its own consumable substance for voltage production.

Lorente de Nó has identified experimentally three distinct fractions of the membrane voltage, the Q, M, and L fractions in order of increasing sluggishness, and corresponding to polarizable systems previously mentioned in relation to electrotonus. They are more or less independently modifiable by stimulation or drug action. The slow L fraction is most nearly related to changes in threshold.

Even though the precise mechanism of membrane voltage remains in controversy, the measurement is often desirable for analysis of drug action, and the equipment is relatively simple. There has been little excuse in the past century for assuming depolarizing actions of drugs before making actual determinations (*cf.* 205, 272).

B. *Properties Associated with Impulse Propagation*

1. *The local excitatory state.* The nature of the process by which electrical or other stimulation gives rise to a propagated disturbance has been the subject of

many investigations (19, 20, 24, 27, 55, 75, 109, 120, 129, 131, 133, 142, 151, 152, 164, 170, 184, 190, 202, 205, 211, 218, 223a, 225, 232, 235, 238, 251, 260, 280, 283, 289).

Following Hill (133), most modern theories are based upon the relationship between a hypothetical local excitatory state (l. e. s.) and an equally abstract threshold state. A stimulus, no matter how brief or small, sets up l. e. s. which thereafter subsides exponentially at a rate dependent upon the electrical characteristics of the fiber. The rate of rise of l. e. s. is likewise determined, and therefore, at least for short stimuli, the state is a function of the duration-voltage product of the stimulus. If the state exceeds threshold, impulse initiation occurs. The limiting stimulus strength to attain threshold with prolonged stimulation was called by Lapique (164) the "rheobase." The time required for a stimulus of twice rheobasic strength to reach the same threshold was called "chronaxie." These two types of measurement, by whatever name, set the time and current characteristics of the l. e. s. It is generally implied in these formulations that the l. e. s. is synonymous with an actual partial depolarization of the membrane, and that the process of depolarization goes to completion when a critical value is exceeded. Since the threshold is itself altered by the stimulus, but more slowly and in a direction to negate the effect of stimulation, a wide variety of phenomena of excitation can be explained by modifications of the time and intensity dimensions of the two factors (*cf.* 27, 146, 152, 225, 232, 238, 251).

It will be obvious from this brief summary that a drug which appears to modify excitability to brief stimuli might do so by altering any or all of four excitation constants. Thus an agent might raise the voltage required for a brief shock, but lower the requirement for a long pulse, if for example, the drug lowered rheobase but increased membrane resistance. Therefore it is of importance to define the stimulating conditions and preferably to use a range of duration before making conclusions concerning pharmacologically-induced "threshold" changes.

2. *Accommodation and the nerve "reaction."* The most general form of two-factor theory assumes an interplay of two processes, one favoring impulse initiation and the other opposing it. Excitation occurs when the first process outruns the second, which is made possible by the relatively greater speed of adjustment of the excitatory process. "Accommodation" is the term ordinarily used for the adjustment of the more sluggish opposing process. If the adjustment did not occur, it would be impossible to account for such phenomena as post-cathodal depression, anodal "break" excitation, etc. There are a variety of methods for the estimation of accommodation, including the use of slowly rising as against rapidly rising pulses, the voltage required for anodal "break" as against cathodal "make" stimulation, or variants of these (27, 133, 218, 232, 251).

From what has been said already, it should be clear that no single accommodation "constant" (251) can describe adequately both the time and voltage course of the process, and the derivation of any single value for drug action will be misleading. Furthermore, asymmetrical effects of anodal and cathodal pulses and anomalous changes with high voltage (184, 232) militate against any simple evaluation. To make matters worse, Lorente de Nó (184) has posed three such processes, or nerve "reactions," corresponding to active adjustments tending to negate the polarizing effects of applied currents, and related in turn to the three basic fractions of the membrane potential. Despite this welter of complexity, it seems important to make at least qualitative pharmacological observation on these processes, since they are intimately concerned with rhythmic behavior of neurones, are labile to drug action, and probably play a vital role in central nervous function.

3. *The action potential spike and the nerve "alteration."* The culmination of all the processes already discussed, and the most obvious function of nerve, is the propagation of a disturbance, the impulse, and its electrical signal, the action potential. DuBois-Reymond (75) first detected the action potential and defined it as a negative variation of the resting potential, and Bernstein (1919) demonstrated that it travelled with a velocity corresponding to Helmholtz' (129) indirect determination of the speed of the nerve impulse. The form and speed of the action potentials of vertebrate mixed nerve were finally determined with great precision by Erlanger and Gasser (82), following the advent of cathode ray oscillography.

Although long considered to be a wave of depolarization, the peak of the action potential was found by Cole and Curtis (49–51, 63) to exceed the membrane voltage by a considerable amount in squid nerve, and all subsequent investigators using microelectrode recording in various tissues have made similar findings (143, 148, 284, 285, 293). The reported amount of overshoot ranges from a few millivolts to almost a complete reversal of sign of the membrane voltage. The external concentration of sodium ion is directly related to the amount of overshoot (143, 148). The phenomenon of overshoot has been variously viewed as the effect of an inductive membrane component (63), a sodium diffusion potential associated with breakdown of the pumping mechanism (143), or the chemical alteration of organic membrane constituents (135).

The form of the action potential in single myelinated fibers has been analyzed by several investigators (82, 184, 233, 243, 263). It is apparent from theoretical consideration (233) that a number of separable processes must be taken into account. There is an initial passive depolarization produced by spread of the electrotonic wave ahead of the advancing spike; a more rapid and relatively linear depolarization associated with the active local process; a slowing of the latter process as the recovery phase takes over even during the rising limb of the spike; a linear recovery of potential almost to the original membrane voltage; a slower recovery phase, the negative after-potential; and finally a prolonged hyperpolarization in the form of a positive after-potential. Extrapolating from data on cardiac muscle, which shows a long phase of relative stability between the depolarization and repolarization waves (285, 293), a similar but brief period of "active" stability is to be expected in other tissues on theoretical grounds, and appears with drugs which retard recovery (270). From these considerations it can be seen that pharmacological studies would profit from a careful measurement of action potential form, preferably made monophasic by crushing between the recording electrodes, since the various components are separately variable.

The height of the action potential is not constant but varies with previous activity and chemical changes in the medium. Lorente de Nó (184) uses the term "nerve alteration" to describe the variable process resulting in the spike, and relates it to active changes in the several components of membrane potential.

The form of the action potential recorded from a nerve immersed in a conducting medium is more relevant to physiology than is the form recorded in air. Lorente de Nó (184) has shown from volume conductor theory and demonstrated experimentally that a positive variation precedes the negative spike, and recordings both from the brain (62) and from peripheral artificial synapses (9, 195, 229, 250, 261) indicate that the initial effect of such an approaching wave upon another separate neurone must be inhibitory.

One of the most striking events associated with the action potential is the fall in membrane resistance, demonstrated by Cole and Curtis (50) for squid fibers, by Hodgkin (141, 142) for crustacean fibers, and by Tasaki (262) for myelinated frog fibers. The membrane resistance drops to about 2% of its resting level at the height of the spike, and recovers slowly during the refractory period. Hodgkin attributes the effect to outward leakage of potassium. Regardless of mechanism, it is obvious that there are profound changes in the physicochemical state of the membrane during activity. It is interesting to note that in heart (285) the resistance fall is associated only with the beginning of the action potential, depolarization persisting long beyond the recovery of resistance. Voltage and resistance are therefore not reciprocally related.

What are the minimum requirements for initiation of a nerve impulse? Lorente de Nó (184) has recorded action potentials after complete anoxia plus poisoning of all known metabolic pathways, simply by elevating the potential of the depolarized membrane above a critical level by means of anelectrotonus. Sodium ion in relatively low concentration (0.015 M) is important for the maintenance of excitability (184), but can be replaced in part by lithium (99, 100), certain quaternary ammonium ions of the type of tetraethylammonium (185, 186), and even transitorily by cocaine (188). Thus only a voltage source and one of several cations for transport are necessary for impulse formation—the rest of the process depends upon chemical interactions in the substance of the fiber.

It should be pointed out that several non-living inorganic systems have been shown to produce action potentials, for example, the iron-wire model of Lillie (174); some of these systems rival in complexity the neurone itself (16, 30). Eyring *et al.* (85) have attempted to set down the requirements for excitation in terms of the theory of absolute reaction rates. Unfortunately they constrict the generality of their treatment by postulating a separation of hydrogen and bicarbonate as the source of voltage.

The traditional controversy between chemical and physical explanations of the action potential continues, but ultimately there must occur some fusion of the two. On the one hand the high temperature coefficients and pharmacological vulnerability of excitation processes speak for the making and breaking of chemical bonds and for enzymatic mechanisms. On the other hand chemical reactions, by their very nature, may be made to produce electrical differences of potential, and there is great convenience in electrical circuit theory both for mathematical handling of data and for practical experimentation. It is of course tempting to assign a vital role in excitation to specific chemical substances, such as acetylcholine (41, 122, 205–207), but the supporting evidence is slim, as will be seen later. If there are trigger substances of low molecular weight which are essential to excitation, they may very well serve as coenzymes in the sense of the formulation given by Welsh (287). In the search for trigger processes, pharmacological research can be of preponderant value. The nature of the membrane change associated with excitation is the core problem of neurophysiology today.

4. *Chemical correlates of impulse propagation.* "Resting" neurones consume oxygen and produce carbon dioxide and water as well as more complex metabolites. A number of investigators have studied changes in metabolic rate during activity and as a result of drug action (28, 32–37, 52, 73, 74, 88, 91, 105, 107, 167, 168, 249). Normally there is an increase of about 20% in oxidative metabolism during propagation of action potentials. Doty and Gerard (73–74) have recently shown that this extra respiration can be abolished by azide, yohimbine, and hydroxylamine, without detriment to conduction of impulses. Conversely, the "resting" metabolism may be separately reduced by methyl fluoroacetate without loss of function. Thus, there is no simple relation between metabolism and activity, nor is it clear why one should be expected. Oxidative metabolism is normally essential to the maintenance of membrane voltage, which is in turn a requirement for excitation. During depolarization or partial reversal the metabolic requirement would actually be lower if it were not for the continuation of processes restoring voltage. Yohimbine actually slows the recovery process, and some investigators (*cf.* 74) have even reported a decrease in metabolism during conduction.

Many investigators have noted an outward flow of potassium ion during activity (54, 89, 142, 155, 156, 246–248, 284, 298), and some have observed a reciprocal inward flow of sodium (121, 156, 284). The actual amounts exchanged are relatively small, but may be of great significance for the mechanism of development of the action potential. Hodgkin and Huxley (142) take the view that the membrane becomes highly and specifically permeable to sodium during the rise of the action potential, and that potassium is passively lost. The inward migration of sodium would be adequate to account for the membrane voltage drop, and the outward flux of potassium for the recovery, according to their view. The ability of released potassium ion to reduce membrane resistance and depolarize could be included as part of the mechanism of propagation. The hypothesis is attractive, but it would be desirable to have suitable data on vertebrate nerve, as well as a repetition of some of Lorente de Nó's extreme tests for dispensability of sodium and potassium before attributing any universality to this mechanism.

Conduction of impulses is associated with liberation of acetylcholine from cholinergic fibers and sympathin from adrenergic fibers, according to the investigations of Lissak (177). Minz (200) and Von Muralt (280) report the liberation of thiamine from sensory fibers during activity, and there has long been conjecture concerning histamine release from unmyelinated dorsal root fibers. To attribute to any of these a vital role in the conduction process in the fibers in which they happen to appear, in the absence of any direct evidence of action of comparable amounts applied externally or injected into fibers, would be to stretch considerably the meaning of chemical mediation. It is of course possible, as has been argued for acetylcholine (205–207), that the important processes occur internally, but more direct proof would be in order. Meanwhile it might be important to search for other organic constituents of nerve which are capable of exerting consistent actions upon nerve (*cf.* 185, 186). There must be many more substances released during activity than have hitherto been accounted for, and modern chromatographic methods might be of assistance in separating them.

5. *Structural changes.* As previously mentioned, Flaig (95), Hill (134) and Tobias (253, 265, 266) have noted visible changes in axones during activity. Hill attributes the decrease in opacity of crustacean nerve fibers following stimulation to an associated swelling, since the two effects have the same course, and can be reproduced in osmotic experiments. There appears to be an initial small and rapid shrinkage and increase in opacity prior to the major change in the opposite direction.

6. *Conduction velocity and failure of conduction.* Once a nerve impulse has been initiated, there arises the problem of its propagation along the axone. This is essentially an explosive process, in the sense that more energy must be liberated by the development of an impulse than is needed for the activation of the next region of nerve. When this condition is fulfilled, conduction occurs without decrement. It is "all-or-none" in the sense that it is independent of the original stimulus, and dependent only on the explosive conditions which prevail locally. The ratio of voltage output to voltage input is called the "factor of safety" for conduction (120), and is in part related to the ratio of total height of the action potential spike to the local critical depolarization required to produce it.

Originally measured for frog myelinated fibers by Bernstein (19) directly and by Helmholtz (129) indirectly, conduction velocity was investigated intensively for vertebrate nerve by Erlanger and Gasser (48, 82, 102) who related fiber size and myelination to function and in turn to speed of propagation. The simplest division of vertebrate nerve groups on this basis is: A fibers: large myelinated somatic motor axones and sensory fibers for touch, pressure and proprioception, of the order of 10 micra in diameter, and conducting with speeds of or up to 100 meters per second in mammals; B fibers: small myelinated preganglionic autonomic fibers predominantly with intermediate conduction velocities; C fibers: unmyelinated post-ganglionic sympathetic and unmyelinated sensory fibers including those for pain, of the order of one micron in diameter and one meter per second velocity of conduction. There is considerable subdivision and overlap within these groups, but the main divisions are of pharmacological importance, particularly with regard to the selective blocking of the smallest fibers by local anesthetics. There are a number of useful reviews which deal with conduction in vertebrate and invertebrate nerve (82, 102, 120, 152, 190, 223, 223a, 238, 288), including theoretical and methodological considerations.

One controversy which has plagued the literature in recent years concerns saltatory conduction in axones with nodes—that is, in vertebrate peripheral myelinated fibers. Bethe (20) first demonstrated that conduction could occur across an injured inexcitable gap, and a number of investigators (81, 101, 151, 260–263, 254) have subsequently presented evidence that the excitation process jumps from node to node, but others (165) find smooth and linear conduction without discontinuities unless the fibers are injured. The question of whether drugs must act on the fiber as a whole or only on the relatively unmyelinated internodes is of course important to pharmacology. Saltation can hardly be a general phenomenon, since central vertebrate myelinated fibers, as well as all unmyelinated and invertebrate fibers in general, are lacking in nodes. But it is important to note that Bethe's original phenomenon, conduction across a nonresponding gap, is of greatest theoretical significance; many studies on transmission across blocks, or between apposed cells, show beyond doubt that the action potential is adequate to excite without the intervention of a mediator (9, 81, 101, 118, 120, 147, 150, 151, 197, 215, 229, 250, 254, 261).

Factors determining conduction velocity have been worked out by a number of investigators (1, 85, 131, 144, 152, 153, 184, 209, 243, 267, 270, 272, 289). Other things being equal, larger fibers conduct faster than smaller, myelinated faster than unmyelinated. The reason for this is implicit in the electrical characteristics of core conductors. Lorente de Nó (184) and Hodgkin and Rushton (144) have proved from cable theory and demonstrated in nerve that the equivalent conduction velocity for a decrementing electrotonic wave is $2\lambda/\tau$, or twice the characteristic length per characteristic time. Actual conduction velocities tend to be of the same order of magnitude. From a glance at our previous discussion on passive properties, a simple relation between characteristic velocity and the membrane capacity and resistance and internal and external resistances can be derived: $V^2 = 4/c^2_m r_m\ (r_e + r_i)$. Thus a larger fiber will conduct more rapidly both because of smaller physical internal resistance and the physiological drop in membrane resistance; a more myelinated fiber will conduct more rapidly because of the preponderating drop in capacity. A non-electrolyte medium of high resistance should and does slow conduction (153, 184). Agents which increase or decrease membrane permeability should have corresponding effect on velocity, but these are still to be reported.

Since the above factors concern only the passive aspects of conduction velocity we must deal also with the local explosion, the ratio between input required and output obtained. Hermann (131) predicted that there should be an inverse relationship between threshold and conduction velocity, and Werigo (289) demonstrated it, although the function is not a simple one (267, 270, 272). Eyring *et al.* (85) have dealt with the underlying chemical dynamics of conduction velocity. The size of the action potential spike must also be considered, since an increase in spike amplitude will lead to increased velocity, other things being equal (1, 209, 243). This may be important in relation to sodium lack (148, 184). It is interesting to note that Lorente de Nó (182, 185) obtained extremely low conduction velocities with some quaternary ammonium salts in place of sodium.

From the foregoing, it can be seen that much information relevant to drug mechanisms might be obtained by combining measurements of conduction velocity with other key determinations.

So far we have dealt only with factors altering velocity. What are the limiting circumstances that can result in conduction failure? It is inadequate to describe a drug as blocking conduction without further examination of mechanism. In general it can be said that conduction will fail if the threshold rises high enough or the spike amplitude falls low enough to reduce the factor of safety below 1.0, or if the membrane potential falls below a critical value. Thus, as we shall see later, many local anesthetics, anticholinesterases, centrally acting drugs and other agents may block by threshold increase, after an initial slowing of conduction, while agents affecting the sodium transport system might act by reducing the spike amplitude. Depolarizing agents, such as KCl, various metabolic poisons, and anesthetic gases, can block without producing an initial threshold rise. A depolarizing block might be identified without membrane potential measurement by the unblocking action of anelectrotonus (10, 184, 294–296).

Hyperpolarizing blocking agents are also known, including calcium (184) and very high concentrations of CO_2 (270). Although electrical anodal block is well-known (184, 218, 232, 294–296), hyperpolarizing agents have not been often described. In part the mechanism is probably increase in threshold by increase in the L fraction of the membrane potential (184), more than sufficient to offset the corresponding increase in action potential amplitude. Catelectrotonic relief of block would assist in identifying a hyperpolarization, as would also the relief of block by rapid repetitive stimulation (184).

Other types of blocking mechanisms which are indirectly related to threshold or polarization alterations include prolonged delay in recovery time and failure at high frequency stimulation, as with yohimbine (74), and possibly hyperaccommodation, as with prolonged phosphate treatment (270).

For differentiating the mechanisms of various unknown conduction-blocking agents, it can be seen that several measurements at the time of onset of block are important. With conventional stimulating and recording equipment they include measurement of threshold with long and short pulses, estimation of conduction velocity or at least of stimulus-response latency, tests with slow and fast stimulus frequency, measurement of spike height of individual fibers as well as of total action potential in a mixed nerve, and observations on the relief or exacerbation of block by electrotonus at anode or cathode. In addition, it is sometimes valuable in a blocked nerve to record at the stimulating cathode for evidence of abortive decrementing impulses (152), which are frequently found with threshold-raising agents.

7. *After-potentials and recovery of excitability.* Several investigators (7, 104) had previously noted the existence of prolonged electrical changes after impulse conduction, but Erlanger and Gasser (82) were the first to establish the relationship between these after-potentials and the recovery of excitability. Lehmann (171, 172) and Graham (115, 116) are among those who have studied the effects of pH alterations and inorganic ions on these related functions in vertebrate nerve, while Shanes (246, 247) has made pharmacological observations in in-

vertebrates. Lorente de Nó (183, 184) has reinvestigated the relationship with the aid of drugs in frog nerve.

The classical excitability cycle (152, 190, 238) was considered to consist of an absolutely refractory period concomitant with the depolarization process, and a subsequent relatively refractory period during which threshold recovered toward normal. But the latter period may be very short (one or two msec. in frog sciatic) and continuous with a rapidly developing period of supernormal excitability which reaches a peak at 5–10 msec. in frog sciatic A fibers in air, and more slowly subsides. Certain drugs, particularly veratrine, markedly increase and prolong it. Following this there may be a much longer period of subnormal excitability, more prominent in smaller and unmyelinated fibers, or after repetitive stimulation, and persisting even for minutes under the action of some drugs such as yohimbine. Associated with the supernormal phase is the negative after-potential, and with the subnormal phase, the positive after-potential (82).

Lorente de Nó (184) has reinterpreted these changes in the light of his investigations on polarizability of nerve. The three fractions of the membrane potential, which collapse to varying extents during the action potential spike (nerve alteration), are actively restored in the same general manner as if a cathodal pulse alone had been applied (polarization) and actively opposed (nerve reaction). The time lag of the reaction processes and the tendency to overshoot, multiplied by the interrelations between the three polarizable layers, gives rise to a variety of oscillatory changes in membrane potential. However, it is notable that in general the threshold and the membrane potential behave in roughly parallel fashion in Lorente de Nó's observations as in those of others; and also these changes show great liability to drug action, which increases their interest for pharmacology. Although post-impulse changes may not be of great significance for function in peripheral nerve, they must become of great importance in spontaneously active nerve-nets, particularly in the cerebral cortex.

One interesting aspect of the recovery process is the tendency of spike amplitude to increase during repetitive stimulation, a result of the cumulative loss of membrane potential fractions (184). This effect appears to be of importance for facilitating effects of repetitive sensory root stimulation in the spinal cord (178). It can be imitated in peripheral nerve by strychnine (173, 281) and may account in part for the central excitatory effects of that drug.

8. *Rhythmicity and spontaneous firing.* Rhythmic activity, spontaneous or evoked, is more the rule than the exception in neurones which are left intact within the body, whether they be peripheral or central. Sensory and motor information are communicated usually in trains or bursts of action potential spikes. Most investigators find some spontaneous activity in motor or sensory nerve fibers, and isolated nerve under supposedly optimal conditions usually shows a few discharging fibers. It is probably fair to say that activity, rather than quiescence, is the mode of existence of living tissues, and it is notable that both neurones and effectors, deprived of afferent connections, tend to approach a level of resting spontaneous activity. Therefore a few words might be well spent on such phenomena.

Rhythmicity and the factors concerned in adaptation have been studied in single fibers from sensory receptors (2, 154, and many others). Among the highest frequencies of discharge known are those recorded in pyramidal fibers during convulsive seizures, produced electrically or by convulsant drugs (3). Spontaneous rhythms are characteristic of some invertebrate ganglion cells (221–223) and rhythmicity would appear to be more common in general in invertebrate than in vertebrate neurones in response to stimulation (223, 223a, 238, 288). Rhythmic activity following the use of various "decalcifying" and other agents have been widely studied (8, 34, 37, 42, 114, 171, 184, 189, 193, 203). Among the agents which have been used to produce repetitive firing, Gordon (114) lists: fluoride, carbonate, phosphate, oxalate, citrate, tartrate, malate, acetate, lactate, pyruvate, formate, nitrite, sulfite, thiocyanate, thiosulphate, tetraethylammonium, DDT, naphthalene, hexachloreethane, veratrine, quinoline. To these should be added increase in pH and decrease in calcium ion.

There has been a strong tendency to equate all of these excitatory drug actions with reduction in or competition with calcium (34, 35, 114), but Lorente de Nó (184) attacks this thesis in characteristic fashion, and attributes the actions in most cases to more specific effects on metabolic processes producing the various fractions of membrane voltage.

The occurrence of rhythmic local potentials as a precursor to firing of repetitive spikes has been noted by many (8, 34, 35, 171, 184). Brink *et al.* (34) have correlated these rhythms with the basic electrical characteristics of nerve.

In general, two types of mechanism would seem important for the development of rhythmicity. One is a fall in threshold, which permits small oscillations in membrane potential to become adequate for excitation. The second is a fall in membrane resistance, which by loss of damping would tend to oscillatory phenomena in the equivalent circuit of nerve. The former effect is frequently but not always found. The latter has not been sufficiently studied. Neither explains why local oscillations occur in the first place, although many types of nonliving models may be constructed which oscillate when a continuous source of energy is provided.

One point of pharmacological interest in drug-induced neural rhythms is the great sensitivity of such preparations to various depressant drugs (159, 267–271), in comparison to normal "resting" nerve. Taking advantage of this sensitivity, one can often demonstrate consistent actions at dose levels which approach those for central nervous effects.

9. *States of excitation.* In concluding this general discussion on properties of nerve and effects of drugs thereupon, something should be said about the variations in status of nerve which may cause wide variance in pharmacological studies. Lorente de Nó (184) has taken this variance into account and described several alternative states of excitability, based on the appearance of their recovery curves and after-potentials, and related to differences in the fractions of the membrane potential. These include, for frog nerve, a "resting" state, corresponding to that *in vivo* when 5% CO_2 is supplied or the membrane potential raised by anelectrotonus; a "pseudo-resting" state after a period of time in air

without CO_2; an "exalted" state immediately after dissection; a spontaneous "rhythmic" state seen particularly in mammalian nerve in oxygen without CO_2; and a "depressed" state resulting from fatigue or anoxia. These studies emphasize the important role of CO_2 in neuronal function and the desirability of imitating *in-vivo* conditions for a proper evaluation of drug actions.

III. ACTIONS OF DRUG UPON NERVE

A. *Local Anesthetics*

1. *General considerations.* Cocaine, which had been isolated from coca leaves in 1860 by Niemann, became the first widely used local anesthetic shortly after its introduction in 1884 by Carl Koller. Two decades later the synthesis and clinical trial of "novocaine" (procaine; diethylaminoethyl p-aminobenzoate) by Einhorn and his collaborators in 1905 opened a new era in the search for better local anesthetic agents. Of the hundreds of active compounds which have been tried, procaine still remains the most widely used. Although far from the most potent, it has a high margin of safety between local anesthetic and irritant concentrations.

The desired property of a local anesthetic is its ability to reduce pain by its local effect in blocking impulse conduction preferentially in the pain fibers along a nerve trunk or at their terminations, without the production of irritation, nerve or tissue damage, and without systemic effects of absorption from the local site of administration. By an extension of local anesthetic action, regional nerve block, diagnostic ganglionic block, and caudal or spinal anesthesia are also made possible. In recent years the local anesthetics, particularly procaine, have been found to give analgesia upon intravenous administration, but it is still not clear to what extent this represents an ability to block conduction in small fibers.

One of the most complete studies of chemical and physical properties of the local anesthetics is that of Löfgren (181). Taking xylocaine (α-diethylamino-2,6-dimethylacetanilid) and a number of related substances, Löfgren has examined their physicochemical characteristics as well as various features of their clinical and experimental actions. By determining molar refraction, ultraviolet absorption spectrum and other properties, he demonstrates the effect of alterations in the aromatic ring upon such features as resonance through the ring-attached nitrogen, and of this factor in turn upon thermodynamic ionization constants. He presents data showing the importance of this latter factor on local anesthetic activity, demonstrating that it is the free base which determines the activity. From measurement of the oleyl alcohol/water distribution coefficients he demonstrates that local anesthetic activity does not follow the Meyer-Overton rule. He also derives relationships between chemical structure and rate of diffusion into a nerve trunk, a factor of clinical importance in establishing nerve block. He concludes from these studies that while indifferent narcotics, which adhere to the Meyer-Overton rule, probably act through van der Waal forces rather than chemical combination, the amino groups of the true local anesthetics interact with polar groups in the lipo-protein-metal film of the nerve membrane.

The mechanism of local anesthetic action seems to be essentially an elevation of threshold, the smaller fibers being the most vulnerable. Gasser and Erlanger (102) demonstrated that conduction block produced by cocaine appeared first in the small unmyelinated fibers of a nerve trunk, including presumably those chiefly concerned with the modality of pain, and last in the large afferent and efferent myelinated fibers of fast conduction, the motor fibers being the most resistant of all. This was not the same as the order of block produced by mechanical pressure. Evidence from the laboratories of Bishop and others (17, 25, 184, 272) indicates that nerve block is produced by concentrations below those which cause depolarization of the nerve membrane, and that threshold elevation is the principal factor in blocking conduction. Shanes (246, 247) has presented evidence that the local anesthetics stabilize the nerve membrane against potassium loss. Löfgren (181) has proposed that local anesthetics, in contrast to many depressants which in large concentrations have local anesthetic effects, act by polar association between the amino group of the local anesthetic and suitable polar group in the lipo-protein film of the nerve membrane.

Whether local anesthesia involves only a stabilization of the nerve membrane, without modification of the mechanism by which electrical energy is stored and released by the membrane, is a problem still to be decided. There is evidence that local anesthetics alter nerve metabolism. For example, Sherif (249) reported that procaine and cocaine inhibited respiration of the sciatic nerve of the rabbit. On the other hand, the studies of Larrabee *et al.* (167, 168) seem to indicate that conduction may be blocked by cocaine in concentrations which do not alter oxygen uptake.

For details concerning structure-activity relations and action mechanisms of various local anesthetics, several works should be consulted (80, 112, 169, 181).

2. *Procaine.* Although procaine is capable of reducing membrane potential in high concentrations, it seems clear that the major mechanism of block is an increase in threshold, without a notable change in membrane potential (17, 267). Block occurs in both A and C fibers when the threshold is approximately doubled and the conduction velocity halved. Some investigators still imply that hyperpolarization is a factor, on the basis of antagonistic effects of supposed depolarizing agents (14, 96, 97, 212). Shanes (246) has found that in squid nerve procaine suppresses oscillatory behavior and the negative after-potential before block occurs. In crab nerve (247) he noted that concentrations up to 0.02% (0.8 mM) stimulated oxygen uptake, while higher concentrations inhibited Q_{O_2} up to 50% at blocking levels (about 10 mM). In frog sciatic nerve (270) as little as 0.05 mM can be shown to abolish phosphate-induced hyperexcitability and rhythmicity, as compared with the usual full blocking concentration of 10 mM for the same fibers. For comparison, the customary 1% solution of procaine is about 40 mM, and the dilution used for differential spinal block in man (237) is 4 mM. For intravenous analgesia, plasma levels are of the order of 0.001 mM (35a).

Concerning the differential effects of procaine, although a preferential attack on small fibers is generally assumed (237), the rule is not universal. In rabbit vagus nerve it can be demonstrated regularly that a 1.0 mM solution depresses

A fibers first and C fibers last (84). Qualitatively, procaine is a depressant for all peripheral excitable tissues (112), and may be even more effective on skeletal muscle conduction than on nerve (252). It is therefore not surprising that death from accidental overdosage usually is the result of cardiac arrest (112). The ability of procaine to produce central convulsions is paradoxical, since central neuronal thresholds are found to be increased by procaine, while radiation of evoked discharge is also increased by unknown mechanisms (270). It has been shown that the products of procaine hydrolysis, para-aminobenzoic acid and diethylamino ethanol, oppose convulsant actions but not central depressant or peripheral local anesthetic actions of procaine (226, 227).

3. *Cocaine.* Cocaine differs from procaine in possessing more excitatory components, particularly on adrenergic effector systems (112), but differs very little in its action on peripheral nerve. Gasser and Erlanger (102) showed that it first affected small fibers in peripheral nerve trunks, in contrast to pressure and asphyxia. All investigators seem to be in agreement that depolarization is not a factor in blocking with concentrations at least up to 20 mM, but that threshold increase is the primary mechanism (17, 25, 184, 238). There is probably no direct effect of cocaine on oxygen consumption at local anesthetic doses (249), but both anoxic depolarization and subsequent recovery are delayed (184, 247). In squid nerve cocaine suppresses oscillatory phenomena and the negative after-potential (246). In frog nerve it has little effect on electrotonic potentials or other phenomena which can be electrically measured (184), except as noted above. An interesting and paradoxical action of cocaine is its ability to restore conduction transiently in frog nerve in a sodium-free medium; however, it ordinarily summates with partial sodium lack to abolish conduction (188). Lorente de Nó considers the possibility that cocaine may compete in the chemical processes by which sodium ion maintains excitability, while Shanes (246, 247) postulates a stabilizing mechanism opposing potassium loss.

4. *Xylocaine.* Because of the many pertinent observations on physical properties and dynamics of local anesthetic block which have been made with xylocaine and its congeners, the literature is of importance for pharmacological methodology (80, 111, 181). However, there is little in the published reports to indicate any unusual deviation from the mechanism of action reported for other agents of the same root-structure.

B. *Centrally-active Agents*

For the most part those drugs which are used in therapy for their effects upon the central nervous system are not known to have important effects on peripheral nerve at the doses customarily employed *in vivo*. The study of their *in-vitro* actions on nerve in higher concentrations is of pharmacological significance insofar as such measurements may provide clues to central mechanisms, since basic neuronal properties are more difficult to isolate within the brain and cord. Therefore some passing attention will be given to centrally active agents, with a word in advance that there are few examples as yet of meaningful observations on peripheral actions which have helped to explain the differential effects observed centrally. It is of great theoretical interest that some drugs may alter synaptic transmission by a direct action upon axones rather than upon synaptic endings (35, 231).

Except for the central excitants (Section 9, below), all of the agents described in the ensuing discussion can be characterized as central depressants, in the sense that they diminish some aspect of central nervous function, normal or abnormal. Of these, the most heterogeneous group includes the general anesthetics.

1. *General anesthetics.* So diverse in chemical structure are the many substances which produce central depression or complete anesthesia that it is hard to imagine any single mechanism of action common to all. Indeed, there is no reason to believe that a common action is essential, since there must be as many different ways of reducing or blocking central nervous activity as there are independent physiological parameters necessary for the normal maintenance of function in the central nervous system. However, there has been no dearth of attempts to find a common denominator, and certain physical and chemical features appear with sufficient frequency in anesthetics to merit some discussion. The reader is referred to recent reviews by Butler (43) and Gerard (107).

The Meyer-Overton theory (199) depends upon the rule, particularly applicable to alcohols, that the anesthetic potency is correlated with the oil: water partition coefficient. The theory assumes an ability of lipid-soluble substances to produce changes in permeability and electrical properties within the lipid phase of nerve cells. However, since the critical lipid phase of neurones is still relatively unknown insofar as either electrical properties or affinity for narcotics is concerned, and since many single anesthetics and groups of agents fall out of line, the theory does not have great generality.

Other common physical properties have also been found for certain of the central depressant drugs. Thus Traube (*cf.* 137) advanced the theory that anesthetics alter cell surface properties by their absorption thereupon, as suggested by the correlation between anesthetic potency and ability of many drugs to lower surface tension. This concept has become deeply imbedded in pharmacological literature and is often assumed as a mechanism of action, although it has not been subjected to a rigorous experimental test.

A more recent attack upon the problem of the common narcotic action of widely different molecular species has been made by Brink and Posternak (35). They enlarged upon the investigations of Ferguson (92) who had demonstrated that for equally effective doses of many substances the thermodynamic activities lay within a narrow range of values in contrast to a more than thousand-fold range in aqueous concentration. (Thermodynamic activity is an estimate of the work per molecule required to transfer the narcotic from the pure liquid phase to the unknown phase of locus of action in the narcotized cell, and is derived from vapor pressure determinations.) The Brink-Posternak treatment is more general than the Meyer-Overton theory, in that no assumptions concerning the lipid constitution at the site of narcotic action are required. These authors speculate that equal degrees of narcosis are caused by equal numbers of narcotic molecules in those portions of cells in which narcosis occurs. Unfortunately their rule does not cover all of the experimental situations studied or reviewed; for example, the thermodynamic activities of substances producing nerve conduction block in non-synapsing preganglionic fibers of the perfused stellate ganglion in the cat are not constant, but increase with the activity coefficients. Their work has recently been subjected to critical review by Butler (43). The significance of their work, as of Ferguson's, is that it makes possible a comparison of narcotic activities based on a simply measured property of molecules, and suggests a common but non-specific action of a large number of dissimilar compounds. It also stresses the need for a search for more specific enzymatic or other actions of substances which do not fall within the general group on the basis of thermodynamic activity.

If no common physical feature can be held to account for all anesthetic actions of diverse drugs, the same may be said for chemical aspects, in the sense of actions upon enzyme systems or upon any process of bonding of the drug with any constituent of the cell. In recent years particular attention has been given to the action of depressants upon cellular respiratory enzyme systems of the brain. For a general discussion on the present status of the field of general anesthetic effects upon cellular metabolism, the reader is referred to the review of McElroy (198) who defends the thesis that a relatively non-specific mechanism of reversible denaturation of protein may have relatively critical effects upon certain metabolic processes *in vivo*.

Gerard (107) has reviewed the several theories of anesthetic action, and examined critically the evidence for a blocking action of narcotics on specific respiratory enzyme systems. He marshals the evidence which points to an action of several narcotics on cytochrome b or on an intervening flavoprotein, but warns that this site has been identified solely by exclusion of other portions of this sequence, not by positive evidence at the suspected links.

Even if the metabolic steps affected by general anesthetic agents could be well delineated, it is important to know the consequences of this metabolic interference upon particular functions of neurones in order to know the mechanism of action by which anesthesia is produced. At this level of investigation, the drugs which have been most extensively studied are ethyl ether, ethyl alcohol, the carbamates and the barbiturates, particularly phenobarbital.

Ethyl ether was first studied extensively on nerve by Biedermann (24) who found that it abolished slow electrotonus and the polarizability of the axoplasmic core. Bethe (20) made the interesting observation that certain reactions between dyestuffs and axoplasm which were normally altered by current flow were no longer sensitive to electrotonus after ether had been applied to the nerve. Alcock (5), who made the first modern quantitative determinations on membrane potential, confirmed the depolarizing action of ether. Heinbecker and Bartley (128) found evidence for an increase in accommodation in frog sciatic nerve, which should be properly correlated with a transient initial increase in slow electrotonus, especially at the anode, as described later by Lorente de Nó (184). The latter investigator found that eventually the slow electrotonus decreased, concomitant with a fall in membrane potential. When potential fell below about

15% of resting value, conduction failed. It could be restored by anodal electrotonus. Lorente de Nó found a small increase in excitability prior to block, but Wright (297), working with mammalian nerve, found only that excitability decreased as depolarization progressed. Naess (208) has noted a small stimulating effect of anesthetic concentrations of ether *in vivo* in rabbit motor nerve. Whatever the preliminaries, it is clear that ether must be classified as a depolarizing agent.

Chloroform has been less studied in nerve than ethyl ether, but probably works by the same mechanism. It is said (238) to raise rheobasic threshold with no initial effect on chronaxie, and to slow conduction velocity in addition to its depolarizing action.

2. *Ethyl alcohol.* Schaeffer (238) describes ethyl alcohol as increasing membrane potential while increasing rheobase and chronaxie and decreasing conduction velocity. However, most authors have found alcohol to be a depolarizing agent. Feng (90) showed that the course of depolarization was different from that of potassium, involving a sharp fall at a critical concentration. Wright (297) found that the threshold was progressively increased during the course of depolarization but Gallego (98) has observed an increase in excitability during the early course of depolarization. He finds that block occurs at alcohol concentrations of 1.0 to 2.0 M, when membrane potential has dropped by 12.5 mV. At this point conduction can be restored by anodal polarization, but if the membrane potential loss is permitted to go beyond 20 mV, there is an irreversible loss of conduction and polarization.

3. *Carbamates.* Lorente de Nó (184) found that ethyl urethane (40 mM) had little effect on membrane potential and somewhat less effect than cocaine on slowing of anoxic depolarization and recovery. Crescitelli (56, 58), however, noted that a small but significant initial hyperpolarization (actually a millivolt or less) arose with low doses of ethyl, propyl, butyl and amyl carbamates, and that higher concentrations eventually depolarized. Since the hyperpolarization effect did not change with temperature, he attributes it to a physical surface-action effect. Depolarization rate increased with temperature, in a manner to suggest a metabolic action. It appears from his data that block occurs during the hyperpolarization phase (but not that there is a causal relation). Crescitelli favors a decrease in permeability as the mechanism of block. His records are compatible with a threshold increasing mechanism. The order of conduction block is similar to that of anoxia, with B fibers most sensitive, A intermediate, and C least. Equivalent blocking concentrations (ethyl 210 mM, propyl 70 mM, butyl 18 mM, amyl 6.5 mM) give approximately equivalent reductions of air-water surface tension.

4. *Barbiturates.* The effects of pentobarbital (Nembutal) on peripheral nerve fibers have been studied by Heinbecker and Bartley (128). In frog sciatic nerve the changes included increase in threshold, reduction in amplitude of action potential spike and negative after-potential, prolongation of absolute and relative refractory period, slowing of conduction velocity, and a slight decrease in accommodation. In turtle vagus, the small unmyelinated fibers were most easily

blocked while the large myelinated fibers of fast conduction were most resistant. The concentrations required for these actions on nerve were apparently high in comparison with those needed for central nervous depression, but the findings are compatible with central effects of lower dosages. With smaller doses temporal summation and facilitation were reduced in the excised superior cervical ganglion of the turtle. In this connection Larrabee *et al.* (167) have more recently observed that synaptic transmission is more easily suppressed than fiber conduction in the branches of the stellate ganglion of the cat, and that oxygen consumption is decreased even by non-blocking doses.

Eccles (78) has made precise studies on changes induced by pentobarbital in spinal motoneurones. The threshold is increased in the sense that greater depolarization is required in motoneurone soma for the evocation of a propagated response. This action seems to be associated with an increase in membrane resistance. Similar effects are apparent in Curtis' (62) studies on callosal projections. The principle actions of barbiturates on higher centers seem to be a mixture of increase in threshold and prolongation of recovery time (269).

In our own experience (268, 270) barbiturates including phenobarbital, barbital, pentobarbital and Mebaral produce conduction block in frog sciatic nerve in concentrations of 15–30 mM. However, phenobarbital, in contrast to others, has a distinct protective action against electrically evoked hyperexcitability at concentrations of 1.0 mM, a matter of interest in connection with its anticonvulsant action (see below). Pentothal i. v. may increase the direct electrical excitability of cortical neurones in the rabbit at a time when synaptic transmission is depressed (270).

(For consideration of the possible enzymatic actions of barbiturates on neurones, *cf.* 107, 119, 269.)

5. *Relaxants.* Drugs which produce skeletal muscular relaxation through their action upon the central nervous system have recently received considerable clinical attention. Myanesin (mephenesin) is perhaps the best known of these agents; its central action has been related to a preferential block of small internuncials (*cf.* 269 for literature and discussion). Finkelman and Arieff (94) found no direct action of systemically administered relaxant doses of Myanesin on peripheral nerve or muscle in animals.

The author and his colleagues (270, 271) have looked for common effects of relaxants on properties of peripheral nerve and attempted to relate these quantitatively to clinical and experimental observations. The studies were restricted to three representative "antispastics", Parpanit, Myanesin and benzimidazole. These drugs were found to block conduction in frog sciatic nerve by raising threshold rather than by depolarizing the nerve membrane. Increase of threshold, decrease of conduction velocity and spike amplitude, and prolongation of recovery were found within the concentration range for lethal effects in experimental animals *in vivo*. In lower concentrations all three drugs gave protection against hyperexcitability induced by excessive stimulation or by phosphate treatment. These effects were produced at concentrations equivalent to the relaxant dosages for experimental animals. The absolute potency for any of these effects was greatest for Parpanit and least for benzimidazole. The blocking doses and minimally detectable doses, respectively, for these three agents were: Parpanit 0.10 and 0.007 mM; Myanesin, 5.0 and 0.10 mM; and benzimidazole, 6.5 and 0.30 mM. Although these observations reveal the expected order of potency, they are of little help in interpreting the differential manifestations of action seen clinically and in experimental animals.

6. *Anticonvulsants.* Some interesting actions of anticonvulsants upon peripheral nerve have been described by the author and his colleagues (93, 113, 258, 268–270, 292).When untreated frog sciatic nerve is stimulated excessively and repetitively, the threshold falls to about half and recovers over a period of a minute. Concomitantly, repetitive discharges of A fibers are produced by single shocks. The phenomenon is of interest because of its resemblance to similar effects described by Adrian and Morruzzi (2) in pyramidal cells of the cat during seizures. Pretreatment with a number of anticonvulsant drugs can abolish these effects in relatively low doses. In the cases of diphenylhydantoin the action on nerve is apparent at concentrations of 0.04 mM, and can be demonstrated in limb nerves dissected from mammals which have been pretreated i. p. with an effective dose of an anticonvulsant. In addition to these actions, the excitatory effects of phosphate, citrate and oxalate on frog sciatic are opposed by many of the anticonvulsants in low dosage. Korey (159) has observed this effect of diphenylhydantoin and Mesantoin in squid nerve. It has been shown (93, 292) that in the case of diphenylhydantoin these effects are not directly attributable to permeability changes, since there is no marked effect of the drug upon the exchange rate of radioactive phosphate, sodium or potassium. The protective actions of the clinically effective anticonvulsants against excitatory phenomena in nerve occur at doses which have only trivial actions upon normal properties of nerve, except perhaps for a reduction in polarizability and extent of accommodation (270). Effective doses of various barbiturates, hydantoins, oxazolidine-2,4-diones and acetylureas lie usually in the range of 0.1 to 1.0 mM for those compounds containing a phenyl side-group and unsubstituted ring nitrogens; there is also a rough correlation between these structural features and the clinical effectiveness of the same drugs against grand mal epilepsy. Trimethadione, for example, is ineffective by various tests on nerve, while diphenylhydantoin, phenobarbital, Mesantoin, phenacetylurea (Phenurone) and others are active at 1.0 mM or less.

Conduction block does not occur with the anticonvulsants or their homologs until concentrations of approximately 20 mM are achieved, and many anticonvulsants are not soluble at this level. Saturated solutions of diphenylhydantoin have little consistent effect upon threshold or conduction (0.5 mM), but saturated Phenurone solutions (3.0 mM) raise threshold somewhat. Those agents which block conduction at higher concentrations probably do so by threshold increase.

It should be pointed out that many other drugs which are not clinically useful anticonvulsants also have protective actions against chemically or electrically induced hyperexcitability and hyper-responsiveness, but the margin of safety between these effects and actions upon normal parameters of nerve appears particularly high among the anticonvulsants which are clinically useful against grand mal.

7. *Analgesics.* The peripheral nerve actions of analgesics have not been adequately examined by modern techniques. Schaeffer (238) reports that morphine reduces both chronaxie and rheobase in low dosage, and raises rheobase at higher levels, which would be compatible with the mixture of excitatory and depressant effects manifested by this drug on brain (112). It would be of interest to determine the relative sensitivity of various fiber groups to analgesics.

8. *Cannabinols.* Natural marihuana derivatives and their synthetic congeners are of interest because of their mixture of excitant and depressant properties (112), and the very low effective dosage of some of the synthetic compounds (down to 10–30 micrograms/kg), which is the more surprising in that they are non-nitrogenous substances (see 269). Data on peripheral nerve are lacking except for two highly potent synthetic preparations, which were found to be quite without effect on frog sciatic nerve even in saturated solution (270).

9. *Central excitants.* Of those drugs which produce signs of excitement or frank convulsions by central nervous action, Adrian and Morruzzi (3) found that strychnine, picrotoxin, nikethamide, absinthe, and Metrazol all produced high frequency discharges in pyramidal cortical cells of the cat. However, the response seems to be characteristic of seizure discharges however produced, and when we turn to peripheral nerve we find little evidence for anything that can be construed as an excitatory action, except perhaps in the case of strychnine.

Strychnine. Heinbecker and Bartley (127) found decreased threshold and a reduction in degree of accommodation in strychninized frog sciatic nerve. According to Schaeffer (238), chronaxie and rheobase are reduced (but the latter is increased in higher dosage), conduction velocity is reduced and membrane potential is unchanged. In our own experience (270), strychnine produces a moderate increase of threshold and slowing of conduction velocity at a concentration of 30 mM, and no consistent action at 6 mM or below. Wall (281) and Lettvin (173) emphasize the ability of strychnine to increase the amplitude of the action potential spike. This is an interesting example of the possible role of responsiveness rather than of threshold, and the occurrence of such an effect at neuronal terminations on nerve cell bodies would help to explain the known selective excitatory central actions of strychnine (76, 77) in the absence of any apparent direct effect of strychnine on central neuronal thresholds (270).

Metrazol. Eyzaguirre and Lilienthal (86) have seen what appear to be excitatory effects of Metrazol on mammalian nerve and muscle. However, in frog sciatic nerve we have seen no excitatory actions up to 20 mM concentration, at which point depolarizing block begins to appear without lowering of threshold (270).

Caffeine. According to Schaeffer (238), caffeine initially decreases chronaxie and increases conduction velocity, but detailed studies are needed.

Amphetamine. Despite its central excitatory effects, which are usually not attended by a reduction in neuronal thresholds, amphetamine appears to be primarily depressant in frog sciatic nerve (270). In 2 mM concentration it stops multiple firing evoked by phosphate, and at 10 mM it begins to elevate normal threshold.

Ephedrine. Chronaxie and rheobase are both increased by ephedrine, according to Schaeffer (238).

Lysergic acid diethylamide. This substance, related to the ergot alkaloids, is remarkable for the low dosage in which mild central excitatory effects have been reported in psychiatric patients (less than 0.1 mg. orally). However, up to 20 mM there does not appear to be significant action on frog sciatic nerve (270).

C. *Autonomic Agents*

1. *Acetylcholine and congeners.* In spite of the claims which have been made for acetylcholine as a mediator of conduction in nerve fibers (205–207), the results of acetylcholine application to peripheral nerve have been most undramatic. Lorente de Nó (182, 184) demonstrated that isotonic acetylcholine chloride could be used in place of Ringer's solution as an inert medium, that membrane potential was maintained in this solution if small amounts of anticholinesterases were added to prevent hydrolysis, and that with the addition of a small amount of sodium ion, 0.022 M, excitability was also maintained. Without esterase inhibitors, acetylcholine produced irreversible deterioration of the nerve, suggesting that acetylcholine ion could penetrate to some extent. Nachmansohn (205–207) contends that acetylcholine and other quaternary ammonium compounds do not penetrate, so that the internal role of acetylcholine in conduction can not be imitated by external application. In this respect the nerve membrane would appear to be a one-way street, because appreciable amounts of AcCh are found in the perfusate of stimulated motor nerve fibers (177). Acetylcholine is also released (by DDT) from various insect nerve cells (267), but does not appear to have a direct effect upon conduction in cockroach axones (228).

Some authors report neuronal actions of AcCh. According to Schaeffer (238), chronaxie is reduced, conduction velocity increased and membrane potential lowered by acetylcholine. Nordqvist (212) states that 0.5% AcCh can relieve a procaine block. It might be supposed that fibers rendered sensitive by exciting agents would be useful indicators of an AcCh effect; this does not appear to be the case with either citrate (34) or tetraethylammonium (270).

Choline and methacholine are also said to be without effect on nerve, and can be used in isotonic solution as Ringer's substitutes (184). Schaeffer (238) states that pilocarpine decreases chronaxie. It might be of value to study this and other muscarinic acetylcholine-like agents on peripheral nerve with modern methods, if only for the sake of completeness.

For critical discussions of the controversy concerning acetylcholine as a universal mediator, the publications of Feldberg (87), Gerard (106), Gilman (110), Lorente de Nó (182, 184), Nachmansohn (206, 207), Prosser (223, 223a) and Welsh (287, 288) are suggested.

2. *Anticholinesterases.* Much of the argument on acetylcholine mediation has centered around agents which inhibit cholinesterase, since in some cases they

have quite dramatic actions upon neural properties. The case is particularly crucial with regard to DFP (di-isopropylfluorophosphate) which is capable of destroying cholinesterase activity irreversibly. The chief disagreement on fact concerns the extent to which axones can behave normally when most or all of their cholinesterase is destroyed.

DFP. The Nachmansohn group (41, 122, 206, 207) first described conduction block in vertebrate and invertebrate fibers treated with DFP. They attributed the block to a failure of recovery mechanisms requiring the destruction of acetylcholine, and assumed a depolarizing mechanism of block. They found that conduction could be restored by washing, and have demonstrated partial recovery of cholinesterase activity under these conditions. They contend that cholinesterase activity is essential to conduction, but that the cholinesterase activity need be only a fraction of normal.

Crescitelli and Gilman (60, 110) showed that, after conduction block by DFP, propagation of impulses could be restored simply by exposing the nerve to air, presumably due to the rapid hydrolysis of the DFP itself. They indicated that under these conditions the cholinesterase activity of frog nerve was zero. Boyarsky *et al.* (33) found that conduction could continue for 6 hours in 3 mM DFP, at the end of which time cholinesterase activity was absent by the most careful tests. We found (267, 269, 272) that with concentrations of DFP high enough to block conduction promptly (25 mM), there was no fall but sometimes a slight increase in membrane potential. The mechanism of block was shown to be a doubling of threshold in both frog and earthworm fibers. (This effect is clearly seen in the prolongation of latency and action potential in the records of other investigators.) The membrane time and length constants of blocked frog sciatic nerve remain normal, indicating that there is no important change in membrane resistance (270). At a much higher concentration than required for block, 125 mM, depolarization begins (272). Although studies with arthropod nerves and synapses are sometimes difficult to interpret, it would appear from the data of Roeder (228), Schallek (239, 240) and Wiersma (290) that DFP blocks both conduction and transmission by a threshold-raising mechanism.

From these considerations one is forced to conclude that nerve conduction can occur normally in the presence of remarkably little cholinesterase, and also that DFP must have effects over and above its anticholinesterasic action. Heymans (132) has given evidence for effects on other systems, and it is of course no revelation to pharmacologists that drugs which have "specific" selective effects in their lowest effective dose range may have a variety of actions when the dose is increased.

Eserine. Bullock *et al.* (41) also found that eserine blocked conduction in parallel with its ability to depress cholinesterase. Lorente de Nó (184) noted that 0.1 mM was sufficient to prevent the slow irreversible depolarization which occurred in frog sciatic in isotonic acetylcholine chloride. Higher concentrations up to 2.0 mM had no further effect. At 10.0 mM, there was a slight hyperpolarization, followed by depolarization associated with conduction block. The block could be relieved by anodal current. In our observations (272) we found that 25 to 50 mM blocked rapidly without depolarization, while hypertonic solutions finally depolarized. The time factor obviously accounts for the difference between these results and Lorente de Nó's. The mechanism of block, when carried out rapidly, consists in a critical increase in threshold. In later studies (270) it has been noted that threshold increase begins to be evident at 1.0 mM, and that 3.5 mM can block conduction when permitted to act for several hours. An interesting phenomenon encountered at much lower dosage is the prevention of spontaneous firing by tetraethylammonium. This protective action of eserine can be seen with concentrations as low as 0.05 mM and may possibily bear some relationship to anticholinesterase activity.

Neostigmine. Prostigmine was found by Lorente de Nó (184) to prevent acetylcholine depolarization in concentrations of 0.1 mM, but apparently by itself it produced depolarization and block after many hours. Both effects are of interest in that they would seem to indicate that neostigmine can penetrate the nerve membrane (*cf.* 11).

3. *Cholinergic blocking agents.* If nerve conduction were based upon one of the mechanisms of transmission which prevail at various synaptic regions, one should expect to find blocking agents for nerve paralleling those for some particular synapse, and the most likely parallelism would be with autonomic ganglia or neuromuscular transmission. Unfortunately the full gamut of blocking agents has not yet been studied, but it can already be concluded that nerve does not show the expected parallelism. However, the nature of the discrepancy is of considerable theoretical importance, as may be seen from the following studies of quaternary ammonium ions.

Tetraethylammonium is a useful and effective autonomic ganglionic blocking agent. Its pharmacology has been reviewed recently by Moe and Freyburger (201). Among its side-effects are stimulation of peripheral nerve, as noted by Brink *et al.* (34) and others. In our experience this effect is seen clearly at 10 mM and is evident even at 1 mM concentrations. The effect consists of lowering of threshold and the appearance of rhythmic evoked discharges and spontaneous firing, together with slowing of velocity and broadening and blunting of the spike, in A fibers of frog sciatic nerve. The effect can be abolished by relatively low doses of eserine (270).

Lorente de Nó (185, 186) observed that isotonic tetraethylammonium maintained the excitability of most of the B and C fibers of frog sciatic nerve, in addition to maintaining the membrane potential of the nerve trunk, in sodium-free solution. Furthermore, tetraethylammonium could restore excitability of most B and C fibers when conduction had previously been lost, for example, in choline chloride solution. Those fibers which could be restored, designated as "Et" fibers, could also be affected in similar manner by certain other quaternary compounds.

Other quaternary ammonium compounds. Lorente de Nó (185, 186) studied the structural requirements for tetraethylammonium-like compounds capable of replacing sodium. He concluded that quaternary ammonium derivatives having two alkyl substituents ranging in length from ethyl to butyl were adequate. He also concluded from these studies that chemical reactions resulting in a change of tricovalent to tetracovalent nitrogen play an important role in the establishment of electrical double layers in the nerve membrane. Ethylated quaternary ammonium derivatives of piperidine, *l* (+) lysine and histamine were found to play the same role as tetraethylammonium. Quaternary bases extracted from ox brain were even more effective in that they effected a partial restoration of A fibers.

The implications of this work are so important that it would be well to keep in mind the relatively high concentrations of sodium-substituents used, and the need for more convincing demonstration that organic ions rather than sodium itself are primary in the excitation process.

As anticlimax, it might be pointed out that the bis-trimethylammonium compounds *C10*, a neuromyal blocking agent, and *C6*, a ganglionic blocking agent, are without apparent effect on frog sciatic nerve in concentrations up to 30 mM (270).

Of other related blocking agents *curare* has been known since the time of Claude Bernard to be without direct effect on nerve (238), although it may modify nerve excitability curves dependent on muscular responses *in vivo* (257). Nicotine has interesting actions on invertebrate ganglion cells (221, 239, 240, 290); it is not clear whether fibers are directly affected. *Atropine* has mild local anesthetic actions, which is not surprising in view of the resemblance of its structure to that of cocaine (112). The blocking action of atropine is said to be of the hyperpolarization type (97), but it is also reported to have no effect on membrane potential or, for that matter, on threshold (238). Since atropine, in addition to its antimuscarinic actions, has been repeatedly shown to antagonize the effects of cholinergic drugs on the central nervous system (87), it would be of interest to study some of the aforementioned effects of cholinergic agents on peripheral nerve in the presence of non-blocking concentrations of atropine.

4. *Inhibitors of acetylcholine synthesis. Botulinus toxin*, which produces a curariform paralysis of slow onset, is one of the most potent neurotropic substances known. Taking the molecular weight as approximately one million and the lethal dose in rabbits as about 0.05 microgram per kg. (6), the equivalent concentration would be 5×10^{-11} M. Guyton and McDonald (126) estimate the lethal dose as one molecule per 10 neuromuscular end plates. Either the molecule must make many trips, or more probably it sets up a chain of antibody reactions which produce the same end result. In any case it qualifies as a "destructive enzyme". It is not a lecithinase, as are some of the potent neurotropic poisons (6). Both Ambache (6) and Guyton and McDonald (126) have presented convincing evidence that botulinus toxin does not act upon the endplate region of the muscle to produce a curare-type of neuromuscular block. Neither does it block conduction along the nerve trunk. Since the cholinergic effector cells, somatic and visceral, still respond normally to injected acetylcholine, these authors conclude that there is inhibition of acetylcholine synthesis or release from nerve. They also believe that *tetanus toxin*, although it produces predominately opposite clinical manifestations related to acetylcholine overproduction, is

fundamentally related in mechanism of action to botulinus toxin. It is notable that neither of these toxins, in spite of profound intraneuronal effects on acetylcholine metabolism, alters conduction in nerve fibers.

5. *Epinephrine.* Epinephrine has not received attention in axonology comparable with acetylcholine, although an adrenergic substance is known to be released from sympathetic post-ganglionic fibers throughout their length (177). It is said to reduce chronaxie of peripheral vertebrate nerve (238). Since it has both depressing and facilitating actions in invertebrate ganglia (221), and a universal excitatory action on cardiac tissue including that of invertebrates (223a), it has at least as much claim to universality as acetylcholine.

6. *Histamine and antihistaminics.* Barany and Nordqvist (14) have reported that nerve block due to antihistaminics and to procaine can be relieved by histamine. Crescitelli and Geissmann (59) have repeated their observations with a group of ten assorted antihistaminics and other related agents. They found no relation between antihistaminic potency and blocking dose, which varied from 0.8 to 10.0 mM. The mechanism of block was apparently threshold increase without depolarization, although all could depolarize in high concentration. Histamine was without effect up to 30 mM at which concentration it showed slight depolarizing action. They attribute the results of Barany and Nordqvist to acidification by histamine. Nordqvist (212) states that both acetylcholine and histamine (in relatively high dosage) relieve procaine block, and that the effect is independent of pH. Fleckenstein and Hardt (97) discuss the resemblance between anodal polarization and block by calcium, antihistaminics and atropine.

From the foregoing discussion of autonomic agents, it appears safe to conclude that nerve conduction, although undoubtedly dependent on chemical processes, can not be fitted into any of the schemes of chemical mediation which have hitherto been devised for ganglia and neuroeffector junctions. Much of the data suggesting particular systems is apparently based on diffuse effects of high drug concentrations, and does not meet the rigorous criteria previously required for transmission at other sites. Nor is there any *a priori* reason why all fiber types should operate by precisely the same chemical mechanisms, provided only that they are equipped with some general explosive system. The search for new types of systems is still in the embryonic stage.

D. *Metabolic Agents*

Although the purpose of this review does not extend to a discussion of nerve biochemistry and metabolism, it is appropriate to summarize some of the changes which are brought about by excess or deficiency of metabolites or by metabolic poisons.

1. *Oxygen.* It now appears that oxygen is the one substance which is completely indispensable to nerve for the maintenance of its membrane potential, the necessary metabolites being included in the structure of nerve (184). Anoxia slowly decreases the membrane potential after a preliminary period in which already oxidized reserves are apparently utilized (45, 88, 91, 103, 106, 157, 158). The oxygen tension must fall to a very low value (approximately 0.5%) before

depolarization appears (184). Diffusion of oxygen is not the limiting factor in determining rate of oxygen utilization, but rather an internal regulatory process which varies with activity and treatment (91). The oxygen utilization is low compared to most tissues—for frog nerve, values of 0.10 cal/gm/hr. at rest and up to 0.18 cal/gm/hr. for maximum activity are given (106). The values appear to be of the same order of magnitude for various fibers at the same temperature (88). The oxygen requirements in activity may be the same or less than at rest (74). In vertebrate nerve the low oxygen utilization of axones may be related to the relative lack of synthetic processes since the bulk of material required seems to be manufactured in the soma and transported down the axoplasm (286). In fact, a calculation of the power dissipation across the membrane resistance at normal membrane voltage would account for the entire metabolism of nerve (270).

Associated with anoxic depolarization, the polarizability of the various components of the membrane potential is altered (184), the action potential may be reduced to half, and the conduction velocity also halved without great change in threshold until the critical point is reached (297), ordinarily at about 15% less than the normal membrane voltage (184). The conduction process is said to be more resistant than the excitation process to anoxic depolarization (40). The rate of depolarization, as well as the rate of recovery in oxygen, is sensitive to a great many substances; many depressant agents actually slow both processes (184, 247).

All the effects of anoxia can be reversed temporarily by anodal polarization (184), indicating that the existence of the membrane potential is the basic work requirement for excitability.

Older methods of measurement of heat production and O_2 consumption (88) have been improved (74) and new methods devised (34–37, 167–168) to the point where there is now little reason for estimating drug effects on Q_{O_2} by indirect means.

2. *Metabolic poisons.* Practically all the known metabolic poisons have been tested in nerve for one purpose or another. The *carbamates*, of which ethyl urethane probably acts as an antidehydrogenase, have already been mentioned (56, 58, 184). They initially hyperpolarize, presumably by a surface action mechanism, and then depolarize by metabolic inhibition. *Azide*, which acts on the cytochrome system, at concentrations of 0.05 to 0.3 mM inhibits "activity" respiration without abolishing conduction (34, 73, 74), and *hydroxylamine* at 1.0 mM has the same enzymatic and nerve actions. *Yohimbine*, at 0.01 to 1.0 mM, similarly affects "activity" respiration (74) presumably through the same system. More important, it brings about a great lengthening of refractoriness (259) and a corresponding increase in positive after-potential (246, 247), and blocks by threshold increase (194). The relationship of these effects to its known adrenergic blocking action is unclear. *Cyanide*, another agent working on the cytochrome system, depolarizes reversibly in concentrations from 1.0 to 50.0 mM in frog nerve (184, 279). It has an initial excitant action on crayfish ganglion cells (222). *Carbon monoxide*, an oxygen competitor in the cytochrome system, in high con-

centrations depolarizes frog nerve (297), and in crayfish ganglia produces a reversible inhibition of spontaneous activity which can be partly abolished by light (222).

Of the glycolytic inhibitors, *iodoacetate* has no effect on crayfish ganglion cells in air (222), blocks glucose protection against anoxia in crab fibers, but by itself reduces the rate of anoxic depolarization (247). However, in frog nerve it is said to increase anoxic depolarization (45). Depolarizing concentrations of 1.0 mM *iodoacetamide* are reversible by anelectrotonus (184). *Fluoride*, which presumably blocks both aerobic and anaerobic glycolysis, at first excites in 20 mM concentration (possibly by its action on calcium), but eventually blocks by depolarization (184). It may account for the irreversible block noted after long exposure to DFP (272).

Methyl fluoroacetate, which acts early in the tricarboxylic acid cycle to cause accumulation of citrate, acts in a manner opposite to azide, reducing "resting" respiration by half while leaving "active" respiration and conduction intact, in a concentration of 5.0 mM (32, 73, 74). *Dinitrophenol*, in line with its general ability to stimulate metabolism, shows only excitatory effects in crayfish ganglia at low doses, but finally depresses in high (222). *Phlorizin*, presumably active on phosphoryllation mechanisms, has a hyperpolarizing action at 10 mM and does not depolarize frog nerve even at 30 mM; at 20 mM it blocks A fibers before C, and the hyperpolarization block can be relieved by cathodal stimulation or anoxia (184).

Oxine (8-hydroxyquinoline), a metal chelating agent especially for iron, produces hyperpolarization and conduction block at 3 mM; *Carbostyril* (2-hydroxyquinoline) and *quinoline*, which are less effective in formation of metal chelate complexes, produce the same effects at 7.0 mM (57). In crustacean nerves, however, quinoline produces spontaneous firing and possibly partial depolarization, reversible by calcium (114). *Thiocyanate* combines hyperpolarization with spontaneous firing, the latter supposedly by the establishment of a sharp voltage gradient (32). *Arsenite* at 10 mM produces depolarization (184), possibly by action on sulfhydryl groups. *Cupric salts* produce irreversible depolarization in frog nerve in doses of 5 to 10 mM.

In general, it can be said that the differential metabolic blocking agents have interesting but sometimes unpredicted actions on the function of nerve cells, and that only to a limited extent do the data suggest roles of particular metabolic enzyme systems.

3. *Metabolites. Glucose* and other sugars in isotonic solution containing adequate sodium ion were found by Lorente de Nó (184) to have no important effects on nerve except those which could be accounted for on the basis of a combined longitudinal and membrane resistance increase due to reduction in conductivity (increased apparent membrane potential and spike height, decreased conduction velocity). However, glucose as well as *levulose* delayed anoxic depolarization, but the nerve suffered an irreversible loss of excitability in spite of restoration of oxygen. The effect was shown not to be due to accumulation of lactate. It could be prevented to some extent by adaptation of the nerve to anoxia prior to glucose

treatment. In contrast, *galactose* and *sucrose* had no such effect. The mechanism of this phenomenon is obscure, except insofar as it illustrates both the utilizability of glucose and levulose and the distortion of metabolic processes when excessive use is made of anaerobic pathways.

Insulin in low concentration is said to excite, and in high concentration to to depress, firing in crayfish ganglion cells, presumably by action on hexose metabolism (221).

Glycerol appears to have no effect, and *acetone* inconstant depressant actions, in isotonic solution on frog nerve during exposure for several hours. *Acetoacetate* prolongs refractoriness but has no other important effect in isotonic concentration (270).

A number of the short-chain acids produce spontaneous firing in frog sciatic nerve, including *acetate*, *lactate*, *oxalate* and *citrate*; *valerate* does not, and it produces a moderate initial increase in membrane potential (184). The possible role of the longer-chain acids in nerve function is a matter of controversy (135, 136, 184).

Various *alkyl amines* appear to produce a depolarizing type of block in crustacean fibers (290). In ability to depolarize frog nerve, the longer-branched alkyl amines approach potassium, while the shorter have little effect (291).

Thiamine is of some interest because of its apparent release from sensory fibers during conduction (200, 280). Isotonic solutions eventually depolarize irreversibly (184), but the onset of the block and depolarization is so slow as to merit some caution concerning the assignment of a role to thiamine in the conduction process.

In summarizing the actions of some metabolites, one is again struck by the high concentrations required to produce any notable effect. Since it cannot easily be denied that they play a functional role, at least in the processes required for maintenance of membrane potential, their very inertness may give comfort to those who contend that other important cell constituents may not have easily demonstrable effects of direct application in excess of normal requirements.

4. *Carbon dioxide.* Waller (282) first noted the effects of carbon dioxide on nerve and called attention to the importance of CO_2 for normal function. Davis *et al.* (66) observed that CO_2 increased electrical threshold, decreased the conduction velocity, and increased amplitude and duration of action potential. Gerard (104) observed prolongation of the positive after-potential, and Lehmann (171) demonstrated the importance of CO_2 in mammalian nerve for maintenance of viability and exhibition of properties equivalent to blood-perfused nerve. Graham (115) studied the relationship between after-potentials and excitability in relation to CO_2.

Among additional actions of 5% CO_2 summarized by Lorente de Nó (184) are: enhancement of action potential amplitude and excitability after activity; prevention of decrease of membrane potential and spike height following tetanic stimulation; prevention of spontaneous firing induced by many drugs; restoration of conduction after critical depolarization in KCl or veratrine; and moderate increase in membrane potential. Lorente de Nó points out the resemblance of all of these actions to anodal polarization, and shows that they are attributable primarily to an increase in the L fraction of the membrane potential. He presents convincing evidence that the effects are due to CO_2 *per se* and not to a fall in pH or to an alteration of calcium complexes. He shows also that the effects can occur in the absence of oxygen.

Lorente de Nó intimates that even 100% CO_2 does not impair nerve, but this has not been our experience; pure CO_2 rapidly doubles the threshold and the conduction time, and the effects are not always reversible even after a moderately short exposure (270). To attribute these actions to anoxia rather than to excessive CO_2 requires some rearrangement of time scales, in view of the known slow course of anoxia.

Laget and Legouix (162) have recently made a systematic study of differences in response to CO_2 among various nerve types, using an indirect method for estimation of the L fraction of the membrane potential. While motor roots are relatively invulnerable to CO_2 lack, certain peripheral mixed branches in mammals soon go into spontaneous firing and deteriorate irreversibly in air. Gernandt (108) has illustrated the exquisite sensitivity of post-tourniquet paresthesias in man to changes in CO_2 tension.

Davenport (65) has demonstrated that drugs which suppress carbonic anhydrase activity have no effect on function of either central nervous system, where the enzyme system is present, or of peripheral nerve, where it is negligible.

5. *Hydrogen ion concentration.* Lorente de Nó (184) has indicated that nerve is relatively unresponsive to changes in pH between the limits of 5.5 and 8.0, and that therefore the effects of CO_2 and of many acids within this range are specific rather than related to hydrogen ion concentration. Below pH 3.0 there is conduction block (184), and above pH 8.0 spontaneous firing develops (171). Although this range offers considerable latitude, to be on the safe side we have found it convenient to use a small amount of phenol red in all working solutions and to maintain pH at approximately 7.3 for most observations unless specifically desired otherwise (270).

E. *Inorganic Ions*

1. *Monovalent cations. Sodium* plays a vital role in the maintenance of excitability of nerve, as has already been discussed. For frog nerve, a concentration of 17 mM is adequate to maintain normal function as long as the osmolarity (110 mM) is maintained by such "neutral" ions as acetylcholine, choline or methacholine (184, 187). Many quaternary ammonium compounds having at least two ethyl groups (*e.g.*, tetraethylammonium) can substitute in part for sodium (185, 186). In myelinated nerve the size of the action potential spike varies in a fairly direct manner with sodium concentration (148). Sodium entry into invertebrate nerve during stimulation can be demonstrated (121, 155, 156). Metabolic work is probably required directly for the extrusion of sodium, and the mechanism of extrusion may break down concomitantly with the rise of the action potential (143).

Lithium is able to substitute temporarily for sodium as far as conduction is concerned, but it has different effects on electrotonic potentials and accommodation; lithium also has an initial hyperpolarizing action on the membrane potential, but eventually depolarizes, in part irreversibly (99, 100, 238).

Ammonium ion is unable to substitute for sodium (99, 100, 184); it depolarizes but also may to a limited extent delay the onset of nerve block in the absence of sodium.

Potassium has probably been studied more than any other single agent for its effects on nerve (2, 10, 19, 24, 32, 44, 45, 54, 89–91, 99, 116, 137, 139, 141, 142, 148, 155, 156, 163, 172, 184, 191–193, 221, 238, 246, 247, 270, 272, 275, 276, 298, and many others). The amount of attention is attributable to the universal depolarizing action of potassium, and its unique role as the major intracellular cation. Bernstein (19) postulated that potassium was the only ion of importance that could diffuse freely through the membrane, and therefore establish a potential by concentration difference. Bishop (26) and Höber and Strohe (139) were among the first to study KCl block, and Biedermann (24) had observed that during KCl block excitation could still occur at the anode (break-shock). Various authors (54, 89, 90, 246, 247) have described a simple logarithmic relation between membrane voltage and the inside/outside K ratio, but the ratio is not constant, since inside K tends to vary with the outside concentration (91, 184). The leakage of potassium from nerve during activity has been frequently noted (141, 142, 155, 156, 234, 298).

Potassium causes a fall in membrane resistance (141, 142), and therefore has been implicated in conduction. It has been postulated as a mediator between end organs and sensory nerve terminations (2). It has been reported both to inhibit (45) and to increase (32) nerve respiration. Mild excitatory effects prior to block are probably seen more in vertebrate (44, 116, 238, 270, 272) than invertebrate nerve (270). The normal ratio in frog nerve is better maintained in Ringer's with 5 mM KCl than the usual 1.3 mM (91). In the course of blocking, A fibers are normally affected first and C's last (184), but the relations are somewhat complicated for various nerves (163). Depolarization is not complete below 50 mM (184). The actions of potassium can best be summarized as depolarization, membrane resistance reduction, and obliteration of electrotonic potentials, all of which effects are reversible by anodal block.

Rubidinium depolarizes about as fast and to the same extent as potassium, while *caesium* is somewhat less effective (90, 99, 184).

Although at first glance the series seems erratic, it will be noted that there is a regular trend in the increasing series of monovalent cations from lithium, which transiently hyperpolarizes, through sodium, which is without action, to a peak depolarizing effect with potassium and rubidinium, tapering off with caesium.

2. *Polyvalent cations. Calcium* has also been extensively studied with regard to its actions on nerve (34, 116, 172, 184, 193, 203, 238, 246, 251, 255, 256, 270, 295), and very often effects of other agents have been ascribed to alterations in the state of, or competition with, calcium (34, 114, 171–172). Woronzow (296) noted that a calcium conduction block could be reinforced by anodal or relieved by cathodal current. The general effects of calcium are equivalent at least initially to those of anelectrotonus, including threshold increase, spike increase, and

slowing of conduction velocity. The block can be relieved at first by anoxia, cathodal current or a train of impulses (184). However, concentrations as low as 10 mM eventually produce depolarization, the A fibers being first affected and the C fibers last. The effect may be irreversible, due to acute swelling and damage to the myelin sheath.

Magnesium acts in a manner similar to calcium, but much less markedly (45, 184, 238). The effects of 10 mM concentration are comparable to those of 5% CO_2, including a small increase in threshold and membrane potential. Magnesium also reduces oxygen consumption (103).

Barium (189) was found to have primarily a depolarizing action in concentrations from 3.7 mM to 43 mM. However, the L fraction is increased, causing abnormally large after-potentials and electrotonic changes. Rhythmicity occurs when the L fraction falls sufficiently. A fibers are much more susceptible than C fibers to barium block (125, 294, 295).

Effects of various metals have been described by Guttman (125) and Woronzow (294, 295).

3. *Monovalent anions. Chloride* is probably not indispensable for nerve function, since it can be replaced by acetate, lactate and other ions with no notable difference except for the tendency toward spontaneous firing in some of these solutions (184).

Bromide has minimal effects against electrically or chemically induced hyperexcitability after as much as ten hours in isotonic solution (270). *Fluoride* has marked effects at 20 mM, causing at first spontaneous firing (possibly related to calcium binding) and later an irreversible depolarization in line with its general inhibitor effect on metabolism (184, 270, 272).

4. *Polyvalent anions.* The most generally reported feature of the polyvalent anions has been production of spontaneous firing, usually associated with an increase in oxygen consumption. The action is usually attributed to precipitation of calcium, although this would not explain all of the cases observed (184). In fact, the number of anions of all types which have been listed as exciting substances (114) lead to the impression that chloride is in a class by itself, a sort of normal "depressant" of nerve function.

Citrate and *oxalate* at 10 mM typically produce a fall in threshold, repetitive spikes following single shocks, or spontaneous activity; a fall in membrane potential is not necessary to the phenomenon. Azide, concomitant with a reduction in Q_{O_2}, can depress the spontaneous activity without blocking conduction; KCl, which may increase O_2 consumption but depolarizes the nerve, also stops the discharge; thiocyanate, which increases membrane potential, may increase the discharge rate (34). Carbon dioxide abolishes the excitatory effects of citrate or oxalate (184). One mechanism projected to explain these effects is a lowering of the Q/L fraction of the membrane potential, which would favor oscillatory phenomena (184). Oxygen consumption is increased during citrate or oxalate induced hyperexcitability. *Sulfate* (184) and *phosphate* (268) are able to induce repetitive firing. Phosphate (isotonic) can be opposed by a variety of drugs, particularly the anticonvulsants, in relatively low dosage. Phosphate hyperexcitability can still be demonstrated with concentrations as low as about 10 mM.

Phosphate hypersensitivity is of some interest because of the possibility that metabolic phosphorylation mechanisms are concerned. Furthermore, since anticonvulsant drugs can block the action of phosphate (268), the question arises whether they do so by altering the permeability of the nerve for phosphate. Since P^{32} uptake into various fractions of nervous tissue can conveniently be followed (29, 117, 204, 236), the question was examined in frog sciatic nerve, diphenylhydantoin being used as a convenient blocking agent (93, 292). It was found that diphenylhydantoin had only minor effects on either total uptake of P^{32} or integration into the protein, lipid, organic acid-soluble or inorganic fractions, despite complete protection against the phosphate-induced hyperactivity, so that changes in membrane permeability or synthesis into the major nerve fractions could not be invoked as blocking mechanisms. Therefore, the phosphate hypersensitivity must be both induced and blocked at a point in metabolic processes beyond the initial active transport across the membrane and synthesis into tissue constituents. Further studies with sodium and potassium exchange indicate the possibility that the crucial point might be related to the system involved in the active extrusion of sodium.

F. *Miscellaneous Substances*

1. *DDT and other insecticides.* DDT (2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane) has been shown to produce persistent repetitive firing in crustacean axones in concentrations as low as 0.014 mM (114). The firing is suppressed by increased calcium, magnesium and potassium ion, the latter apparently by a depolarization mechanism. Repetitive firing due to DDT and other agents is inferred to result from a delay in restoration of calcium ion to a surface complex, following the breaking of the chelate linkage of calcium ions to surface polar groups by the initial exciting impulse. That some deeper mechanism may be involved is suggested by the finding that acetylcholine is released by DDT from various neurones, an action not duplicated even by anticholinesterases (267). DDT repetitive firing is not seen in squid nerve, in contrast to crab nerve (246, 247), but excitatory effects have been found in mammalian motor nerve (86).

2. *Veratrine.* Veratrine has long been of interest to neuropharmacology because of its ability to produce vascular hypotension of central nervous origin, and tetanic neuromuscular and ganglionic responses followed by block. On closer inspection these effects appear to be intrinsic in the axone (231) and muscle membrane (161) functions rather than peculiar to neuromyal or ganglionic synapse. The chief effects noted have been a large increase in negative after-potential (145, 161, 184, 231, 242, 246, 247), particularly after repetitive stimulation, and finally a depolarization (90, 96, 184, 238). The negative after-potential may develop a distinct rising phase of its own (161, 184, 242). The blocking effect can be increased by potassium (184, 247) or antagonized by carbon dioxide (184), anodal current (96, 184), calcium (247) and local anesthetics (96), but the unblocking agents also tend to increase oscillatory phenomena. Veratrine increases nerve oxygen uptake (242). Lorente de Nó (184) has given approximately

the following interpretation of veratrine action: Initially or in low dosage (1:200,000) the rate of recovery of the Q fraction of the membrane potential is slowed because of an enzymatic action leading to wasteful diversion of oxidative metabolism; the effect becomes cumulative with repetitive stimulation, and can lead to a depolarization block by loss of Q fraction. Unblocking agents such as CO_2 increase primarily the L fraction, increasing the L/Q ratio and favoring slow oscillatory phenomena. At higher concentration (1:50,000) direct depolarization intervenes.

3. *Antibiotics. Streptomycin*, which has been reported to produce specific destructive effects on the eighth cranial nerve and cortical convulsions on direct application, produces a depolarization type of block in frog nerve, but only at very high concentrations, approximately 40 mM (270). The inhibitory effect of streptomycin on neural and glial elements in tissue culture has been studied (15), a matter of interest because of the use of antibiotics to maintain asepsis in neural cultures (219, 220).

Sulfanilamide and *thiophene-2-sulfonamide* are reported to delay anoxic depolarization and recovery in frog nerve and to interfere with CO_2 effects on membrane potential (245). The latter agent is a specific inhibitor of carbonic anhydrase, but has no effect on normal properties of frog nerve which is relatively free of this enzyme system (65).

4. *Others. Menthol* is reported to have a specific sensitizing effect on the end organs of fibers receptive to cold, but not on other types, thus explaining the well-known subjective cooling effect of this agent (130). *Chloropicrin* has interesting veratrine-like effects on nerve at a concentration of about 1.0 mM whereas *bromopicrin* has an excitant action on ganglia while blocking nerve conduction, apparently by a depolarization mechanism (12).

IV. DISCUSSION

A. *Principal Actions of Drugs on Nerve*

There is a large gap between our original outline of basic properties of neurones and our recital of the empirical findings with particular drugs. In most cases it has been almost impossible to draw the proper conclusions from the data concerning anything more than the main mode of production of block or of excitatory effects. For example, the only agent concerning which a confident statement can be made regarding effects on membrane resistance is potassium, even though we would strongly suspect that citrate, oxalate, phosphate, etc., could be shown to decrease membrane resistance, and calcium at least to raise it initially.

Since we cannot make an ideal classification, it seems useful to set down an empirical grouping based on the typical actions of well-known agents. Such a catalogue has the practical advantage that in laboratory work the standard compounds can be carried along in the same experimental design, for close observation of similarities and differences (a useful rule in any field of pharmacology where new drugs are to be explored or old ones reexamined with new techniques). With this practical consideration in mind, certain main classes of drug action on peripheral nerve come into focus:

1. Depolarizers

a. *Potassium-like:* by virtue of ability to depolarize and to reduce membrane resistance, diminish threshold moderately, produce loss of slow response to anelectrotonus, etc.

b. *Anoxia-like:* because of ability to depolarize slowly in a manner that can be strictly reversed by anelectrotonus.

c. *Ether-like:* with respect to rapid depolarization which can be carried to a point of irreversible loss of electrotonic changes.

d. *Veratrine-like:* with regard to preliminary prolongation of negative afterpotential, especially after rapid stimulation.

2. Threshold-raisers

a. *Procaine-like:* in that membrane potential is essentially unaltered, and large fibers of limb nerves are most resistant.

b. *Carbon-dioxide-like:* by reason of moderate increase in membrane potential, increase particularly of the slow electrotonus, and post-tetanic increment of action potential and excitability.

c. *Calcium-like:* because of producing both hyperpolarization block and ultimate irreversible changes, with preference for large fibers.

3. Excitants

a. *Citrate-like:* on account of threshold decrease, evoked rhythmicity, spontaneous firing, etc.

b. *Tetraethylammonium-like:* specifically through ability to substitute in part for sodium.

4. Inert

a. *Choline-like:* electrolyte which does not alter normal properties in isotonic solution in presence of adequate sodium, oxygen and CO_2.

b. *Sucrose-like:* non-electrolyte, otherwise maintaining normal properties as above.

The classification is incomplete and possibly in error in some respects. However, it might have some real value in improving the lines of communication between investigators, supplementing the extremes of endless recital of protocols by some, of mathematical preciosity by others, and of single-measurement decisions by most. Whether it would be of any value in relating axonological data to fundamental processes or in alerting the research worker to useful pharmacological tools is another matter.

B. *Drug Interrelationships*

The discrepancy in many cases between drug actions on specific systems, such as brain or ganglia, and their lack of action, or inverse or irrelevant action on axones is a strong temptation toward eclecticism in pharmacology. Nevertheless there is no dearth of attempts to develop unifying concepts of drug action (4, 18, 36, 47, 92, 107, 112, 114, 137, 149, 180, 184, 196, 205, 269, 287). The author finds it convenient to classify substances on the basis of the following major considerations:

1. *Non-bonding agents*, of which the heterogeneous general anesthetics and other chemically sluggish or inert compounds form the main bulk. These constitute a group of relatively low potency, acting by Van der Waals forces rather than formation of chemical bonds, concentrating in lipid phases, or at interfaces, cluttering the living space of important enzyme systems and thus disrupting physiological regulations by their physical presence. By their very nature they must have diffuse and universal, but not necessarily common effects. Here will be found particularly those agents whose important effects are upon permeability.

2. *Single-bonding agents*, having at least one reactive group capable of forming one bond at a time with tissue proteins including enzymes, disrupting reactions somewhat indiscriminately, but showing broad groupings based on their reaction types and therefore having physiologically qualitative actions by quantitative preference for certain enzyme types. Such agents as anticonvulsants and sedatives might fall into this class, where considerable variation in non-reactive structure has only trivial effects on potency. Here will be found many agents of intermediate potency capable of altering metabolic mechanisms which maintain or restore membrane potentials and thresholds.

3. *Multiple-bonding activating and blocking agents*, including all those agents related to chemical mediators, known and unknown, which have a spatial relationship of reactive groups guaranteeing the formation of several bonds at a time with very specific tissue constituents. If these constituents are portions of enzymes which activate important reactions only in the presence of a coenzyme, and if the drug fits the description of the coenzyme, then the drug itself may substitute for better or worse as the mediator or activator in processes which transfer energy out of all proportion to that of the bonds formed. If by a small change in structure the drug can still bond but now stands in the way of substrate-enzyme linkage, it must inhibit rather than activate. Since the structural requirements for activation will necessarily be more rigorous than for blocking, probability considerations will favor the decision that a potent new drug will eventually be accounted for as a blocking agent. Since tissues have a common chemical origin, ontogenetically and phylogenetically, it will not be surprising to find, on the one hand, that there are many related enzyme systems, differing in apparently trivial respects in their optimal requirements for coenzyme structure; on the other hand, the minor difference may determine that at one site or in one species a particular drug will imitate a chemical mediator, that at another it will be a powerful blocking agent, that at a third intermediate site it will be weakly activating in low doses and blocking in higher and that at another site it will be relatively inert. With peripheral nerve the issue is forced by the problem of the inertness of acetylcholine, and the excitatory role of quaternary ammonium compounds. If there are indeed chemical mediation steps in some nerves, the mediators may not be far removed in structure from common agents which are active at other sites but inert with respect to axonal function.

4. *Antigens*. By multiplying the size and complexity of a substance to the point

where it begins to resemble an enzyme in specificity of pattern, there is achieved a structure capable of diverting the prevailing synthesis of enzymes, causing new structures to be formed about it, which in turn serve as templates for the building of facsimiles of the intruding substance, and so on in reciprocal fashion far beyond the effect of the original strange molecule. This process comes close to the central avenue of growth itself, and it is probably not completely absent in axones of nerve cells, low though their level of synthesis may be. By looking in this direction we can accept the fact that a substance like botulinus toxin can alter or abolish acetylcholine synthesis in nerve out of all proportion to the original quantity of toxin present. But the possibility is also implicit that by developing new toxins in favorable hosts to chosen fractions of nerve it may be possible to test in a highly specific way the functions of neuronal constituents.

C. *Translation from Peripheral to Central Neurones*

At the moment, most axonology seems considerably removed from problems of central nervous function, except by broad analogy. However, the application of microelectrode stimulating and recording to spinal motoneurons has already settled questions which could have been definitively answered in no other way (79). Neuropharmacology should be able to exploit these advances at other levels of the central nervous system. The beauty of the method is that it permits examination of a single cell in its natural society of cells. With certain technical refinements it should be possible to make measurements of membrane resistance and threshold, as well as of membrane resting voltage and action potentials, at many sites and with many drugs. Except for estimation of cell size, most of the advances of peripheral neurophysiology can be carried to the central nervous system. It is probably by this direct attack, rather than by constructing model nerve nets from peripheral systems or rationalizations of discrepant drug actions such as we have just concluded, that axonologists can contribute most to an understanding of brain pharmacology and physiology.

D. *Perspectives*

The discussion has already indicated a number of directions in which peripheral neuropharmacology is likely to go, both methodologically and theoretically. There is one more which seems pertinent to a conclusion, and that is the need for a more dynamic view of neural mechanisms than now prevails.

Of the two most complete theories available, that of Lorente de Nó (184) fails to account adequately for the phenomenon of action potential overshoot, now known to be present in all excitable cells which have been studied. That of Hodgkin (143) permits a mechanism of overshoot but does not account adequately for either the recovery or for the maintenance of a membrane potential in the absence of sodium. Both theories suffer from a lack of continuity between the "resting" and "active" states.

Now we are forced by certain facts to wonder about the relationship between these states. The data of Doty and Gerard (74) indicate that there need be no greater utilization of energy in one than in the other. Furthermore, we have the example of the long period of hyperdepolarization in the heart (293), and the fact that the heart can be shocked from "activity" into "rest" as well as the other way around (285).

To some extent these facts could all be reconciled if it were assumed that in excitable tissues there are two oppositely directed processes capable of establishing voltage differences across the membrane, and that the products of each are in part the reactants of the other. An observed steady voltage across the membrane would then indicate that their rates were equal, but would give no indication of the absolute rates. If there could exist, as there should in such an autocatalytic system operating between limits, two possible states where the rates were equal and where a forced increase in one process could not be sustained by a corresponding increase in the other (that is, two different states of regulation), then there would have to exist an intermediate state where rates were also equal but an increase in one process led to a greater increase in the other. This intermediate state would in effect be the threshold and, above or below it, the system would have to gravitate to either extreme.

If the membrane voltage in turn were a determinant of the rate of each component process, it becomes possible for an extreme state to develop progressively the conditions for its own abrupt disappearance. Some such mechanism must be taken into account in any theory of recovery or of oscillatory behavior.

Without pursuing mechanisms further, the point to be made is that it is possible to conceive of a reciprocating system in which the same processes which appear so dramatically in the explosive phase and recovery are also operative at all other times, but are in balance.

The value of such a formulation for neuropharmacology would be chiefly this: that it would encourage the search for substances and key reactions involved in voltage production both at "rest" and in "activity" and would relieve the search for elusive "trigger" substances which appear and play a role only during the action potential spike.

Whatever view may be taken of the nature of fundamental processes in nerve, it seems to this reviewer that the continued investigation of effects of drugs on axonal properties, and the use of drugs to assist in the search for basic mechanisms of neural behavior, can continue to be of value even though the principles and practice thus derived will probably find application in more important tissues than peripheral nerve.

V. SUMMARY

1. Some basic properties of peripheral nerve are discussed, together with notes on their measurement and on some typical effects of drugs.

2. The actions of a variety of drugs on peripheral nerve are described.

3. A suggested empirical scheme is presented for the tentative classification of drugs acting on peripheral nerve.

4. Interrelationships between drugs in several categories of mechanism of action are discussed, with particular attention to the contradictory actions of presumed chemical mediators and blocking agents.

5. The applicability of modern peripheral nerve methodology to brain neuropharmacology is noted.

6. Some speculations are presented on the continuity between "resting" and "active" functions in nerve.

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